INDUCTION AND EVALUATION OF TRIPLOIDY IN *CRASSOSTREA MADRASENSIS* (Preston)

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I hereby declare that the thesis entitled "INDUCTION AND EVALUATION OF TRIPLOIDY IN *CRASSOSTREA MADRASENSIS* (Preston)" submitted by me for the Degree of Doctor of Philosophy in (*Zoology*) is the result of my original and independent research work carried out under the guidance of (*Dr.P.Muthiah, Principal Scientist, TRC of CMFRI, Tuticorin*) and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any university or Institution.

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Contents

CHAPTER 1

GENERAL INTRODUCTION

Among the four main groups of aquaculture products of the world, molluscs form the major component, followed by finfish, crustaceans and seaweeds. Molluscs contribute two thirds of the total aquaculture production, and Asia accounts for two thirds of the molluscs produced globally (Nair, 1999). Bivalves are the most important of all molluscs, since oysters, mussels, clams etc., are consumed both as live as well as processed food and are also exported (Samuel, 1988).

The fishery for oyster is worldwide. It is estimated that the world production of oyster is about 1million ton/year. United States of America with 0.3 million ton and Japan and Korea with 0.2 million ton each are the largest producers of oysters (Nair and Girija, 1993). In 2001, the total bivalve trade exceeded 1.2 million tonnes compared to 200,000 tonnes, 25 years ago (FAO, 2003). Of the total world aquaculture production in 2002, molluscs contributed 11.27 million mt. Oysters dominated among molluscs contributing to 36.67%, followed by clams and cockles (29.15%) and mussels (12.2%). The world production of edible oysters in 2002 was 4317,380 t and the important contributions were by China(83.9%), Japan(5.1%) and Republic of Korea(3.9%) (Appukuttan,2005).

Oyster farming occupies a prominent position among the various mariculture ventures. There have been attempts to optimize oyster production by application of genetics and biotechnology through control of development and maturation, gamete production, storage, sex and ploidy manipulation (Wada, 2000).

One of the most important parameters for successful commercial exploitation of bivalves is a need for fast growth. A factor reported to be important in growth, is enzymatic gene heterozygosity. Many studies on growth and enzymatic gene heterozygosity have been carried out in marine bivalves, including oysters (Beaumont, 1982; Mitton and Koehn, 1985; Zouros *et al.*, 1980). Rodhouse *et al.* (1986) observed that multiple locus

heterozygosity was positively correlated with growth rate in young animals, in which tissue production is predominantly somatic in *Mytilus edulis*. In older animals gamete production is greater than somatic tissue production and this correlation between growth rate and multiple locus heterozygosity appears to be replaced by a positive correlation between multiple locus heterozygosity and fecundity (Rodhouse *et al.*, 1986). Two common methods for increasing heterozygosity in aquaculture are hybridization and polyploidy (Allen, 1998).

Triploids, organisms with three sets of chromosomes instead of the normal two sets can be useful and advantageous in shellfish aquaculture. They often have faster growth, improved meat quality and increased disease resistance (Allen *et al.*, 1989). The main benefits of triploid animals arise as a result of their sterility. Since no energy is spent for reproduction, it is reallocated for better growth and glycogen storage. This in turn can add texture and flavour to the meat. Sterility in triploids is because homologous chromosomes cannot synapse in the formation of gametes. From a commercial point of view, sterility is advantageous because the metabolic energy normally utilized in gonadal development, is used for increased somatic growth. Thus, triploids of commercially important oyster and scallop species often grow faster than diploid individuals (Beaumont and Fairbrother, 1991). Allen and Downing (1986) observed that glycogen levels often remain higher and more stable during the reproductive period in triploids than in diploids. They observed 72% increase in dry weight of the triploid *Crassostrea gigas* during reproductive season compared to 34% increase in their diploid counterparts. Glycogen content also remained high throughout the year, which results in constant meat quality and more energy reserves (Dufy and Diter, 1990). The potential for increased growth, meat yield and quality are the main advantages of triploid oysters (Chourrout *et al.*, 1986).

In oyster farming, harvesting and shucking of oyster meat depends on the spawning season of the oysters. To realize higher yield, oysters have to be harvested before spawning, since the meat becomes watery and meat weight decreases after spawning. Triploids, in contrast, do not expend much of their energy on gonadal maturation and spawning (Cox *et al.*, 1996). Triploid *Saccostrea commercialis* and *C.gigas* grow faster than diploids and they are well accepted by consumers and taste panels (Nell *et al.*, 1994; Maguire *et al.*, 1998). Triploids also overcome the problem of poor post spawning meat condition in summer–autumn in Tasmania (Nell and Maguire, 1998). Triploid Pacific oysters maintained a condition index advantage over diploids for more than 200 days after spawning. Triploid Pacific oysters also exhibited a bell shell shape (Nell and Maguire, 1998).

Comparison of growth rates between diploid and triploid *C.gigas* cultured in Fujian (China) shows that shell length, shell weight, body weight and wet meat weight of the triploid were higher by 14.4%, 7.66%, 35.27% and 73.25% respectively than diploids at 17 months' of age. During the reproductive period, body weight and wet meat weight of triploids obviously exceeded those of the diploid (Zhinhan *et al.*, 1999). Wang *et al.* (2000) observed that triploid oysters grew faster than diploids with an increase of 15.56% to 41.89% body wet weight when they reached harvestable stage. The mean production of triploids was 20% higher than that of the diploids. The size of triploids are significantly larger than diploids by 12-30% in *C.virginica*, 25-51% in *C.gigas*, 42-52% in *C.dalienwhanensis*, 60% in *Ostrea edulis*, 41% *Saccostrea commercialis*, 72% in *Mulinea lateralis*, 27-58% in *Pinctada martensii*, 36% in *Argopecten irradians*, 32-59% in *Chlamys mobilis* and 81% in *Chlamys fairrie* (Guo *et al.*, 2001).

Oysters are harvested when the condition of the meat is at optimum level. The oyster condition factor indicates the fullness of meat in the shell cavity and is an important factor to be considered for harvesting. Akashige and Fushimi (1992) stated

that in the spawning season, shell weight, total weight and soft body weight of diploids decreased. Similarly condition index decreased sharply in diploids in the spawning season and this sharp decrease was not observed in triploids. Allen and Downing (1986) reported that gametogenesis in triploid *C.gigas* was inhibited and that carbohydrate content evaluated in the whole organism decreased from previous values in diploids at the maturation peak, whereas triploids showed significantly higher levels of carbohydrates. For the same species, Akashige (1990) observed twice the amount of glycogen in triploids than in diploids. For the clam *Tapes phillipinarum*, a higher meat weight, carbohydrate content and condition index for triploids than diploids has been reported (Utting *et al.*, 1996).

Oysters occur all along the Indian coastline in backwaters, bays and estuaries. *Crassostrea madrasensis* (Preston) is the common backwater oyster found all along the east coast, but along the west coast, it is confined mostly to the southern region. It occurs abundantly in Ennore and Pulicat areas in Chennai, Sonapur in Orissa and the Vembanad Lake in Kerala. *C. madrasensis* is a highly suitable species for culture because it has a fast growth rate and is tolerant to a wide range of salinities. Central Marine Fisheries Research Institute (CMFRI), the premier research institution in India, has supported research on oyster culture for the last 25 years, which has resulted in the evolution of a complete package of oyster culture technology, including hatchery production of seed.

Because of a sessile habit and a comparatively low position in the food web, the edible oyster forms one of the best-suited animals for large-scale culture. Oyster meat is very delicious and also nutritious with appreciable amount of glycogen, proteins, vitamin A and B and minerals. The succulent meat of the oyster has graced the tables of gourmets and gourmands alike. The international export market value of 1 kg of chilled/frozen oyster meat varies from Rs. 125 – 300 (Appukuttan and Muthiah,

1996). The demand and high price of oyster meat in the international market therefore augurs well for the expansion of edible oyster culture in India.

Oyster culture is practiced in many countries to meet domestic demands. In India, it has not yet reached commercial level because of the negligible demand in domestic markets, failure to develop proper processing technology for export abroad (Durve, 1974) and other biotechnological and socio-economic problems (Mohan Joseph, 1993). The present utilization of edible oysters, obtained from a small-scale fishery of the coastal waters and backwaters of India, is mainly for the production of lime from the shell. However, with growing awareness for the need for more nutritious food, demand for oyster meat has risen substantially in the country. Considering the commercial importance of edible oysters, the CMFRI, initiated oyster farming in 1970 through the rack and tray method. These research efforts resulted in development of suitable techniques in seed collection, farming and harvesting of oysters. In 1982, the CMFRI successfully produced oyster seed through a hatchery system, (Nayar *et al.*, 1984; Muthiah *et al.*, 2000). Commercial scale oyster farming was attempted in Ashtamudi Lake, Kerala during 1995-96 and more than 100 tonnes of oysters were harvested after 5-6 months of farming (Appukuttan, 2001). There is also good demand for live shell-on oysters in the international market. Since 1981, oyster shell powder is exported to Arab countries. Oyster shells account for about 85-90% of the total weight of live oysters and contains calcium oxide to the extent of 52-55%, by weight. Shells are used for manufacture of calcium carbide, lime fertilizers, cement and in other lime-based chemical industries. Further, the oyster shells are ideal for use as collectors for the collection of oyster spat. Shells can also be broken to suitable size and used as poultry grit (Rajapandian and Muthiah, 1987).

C. madrasensis attains a mean size of 80-90mm weighing 80-100g at end of one year. The meat forms 8-10% of

the shell-on weight of the oyster. Cultured oysters are harvested depending on a condition factor. The average condition factor ranges from 41.4 during post spawning periods to 78.8 during pre-spawning period (Nayar *et al.,* 1987). Better oyster meat yield is obtained before the spawning season i.e. April-May and August-September. Thus, harvesting is seasonal and depends on the spawning season. To facilitate harvesting of oysters irrespective of spawning seasons with enhanced meat weight and condition factor, production and culturing of triploid oysters has been suggested.

Triploidy can be induced by blocking either the first meiotic division or the second meiotic division. For production of triploids, Stanley *et al.* (1984) suggested that triploids produced by blocking meiotic I division would be significantly more heterozygous than conventional triploids produced by blocking meiotic II division. It has also been reported that heterozygous triploid Pacific oyster grows faster compared to conventional triploids (Yamamoto *et al.,* 1988), because of the enhanced rate of heterozygosity. Therefore, the present study attempted to produce triploid oysters by blocking extrusion of the Ist polar body as well as the II polar body in the Indian edible oyster *C. madrasensis* and compared the relative heterozygosity as well as the performance of both I and II meiotic triploids.

Attempts have been made in the present study to compare the relative efficiency of various triploidy induction methods and to estimate the biochemical constituents of triploid individuals, vis-*a*-vis the diploid individuals.

The objectives of the study were to:

1. Develop suitable methods for triploid induction in *C. madrasensis*;
2. Confirm triploidy by metaphase spread preparation and allozyme pattern;
3. Assess the performance of meiotic I triploids with meiotic II triploids;

4. Compare heterozygosity of both I and II meiotic triploids over controls using vertical polyacrylamide gel electrophoresis; and
5. Evaluate the performance of triploids in growth and biochemical constituents.

CHAPTER 2

BIOLOGY OF OYSTERS

2.1. Classification

***Crassostrea madrasensis* (Preston)**

Phylum :	Mollusca
Class :	Pelecypoda
Order :	Eulamellibranchia
Sub Order:	Ostracea
Family:	Ostreidae
Genus:	*Crassostrea*
Species:	*madrasensis*

2.2. General Characters

Edible oyster is a sedentary animal. The soft body of the animal is encased by two shell valves - a lower cupped valve (left valve) and upper flat right valve (Plate 2.2.1). While the lower valve is cemented to the substratum the upper valve acts as a lid to open and close by a hinge mechanism connecting the two valves. The movement of valve is accomplished by the contraction and relaxation of the adductor muscle. The shape of shell is irregular, covered by numerous foliaceous laminae; the hinge is narrow and elongated; the adductor scar is sub-central remiform and dark purple in colour, the inner surface of the valves are white, glossy and smooth. The shell consists of three layers. The outer most is the periostracum, the inner one is known as the nacreous layer, which is thin, hard and usually shiny and the middle layer is thick and chalky and composed of calcium carbonate.

Plate 2.2.1

Edible oyster *Crassostrea madrasensis*
Upper shell valve removed and Whole oyster

2.3. Food and Feeding

The food of oysters consists mainly of organic detritus and phytoplankters, like diatoms and nannoplankters (Rao, 1974; Rajapandian and Rajan, 1987). Microorganisms and other particulate matter are filtered from seawater by cilliary action of the gills and transported to the mouth and from there passed on to the stomach and digestive diverticula for digestion and absorption (Rajapandian and Rajan, 1987). In tropical countries since light and temperature are relatively constant throughout the year, salinity among other factors, is more important in influencing the abundance of food and feeding intensity of oysters (Quayle, 1980).

2.4. Reproduction

Sexes are separate, although hermaphrodites are not uncommon (Rao *et al.*, 1987). Young oysters function as males (60-75mm) and later become females (Rajapandian and Rajan, 1987). The ovary and testis consist of a series of branching tubules or follicles on each side of the body covering the visceral organs. The ripe eggs and sperms pass along a series of tubules by cilliary action in these tubules, which finally merge in a tube along the dorsal side of the body. Two separate systems of genital canals are found one on each side of the oyster, which open, into the epibranchial chamber and from there gametes are discharged.

In one spawning act, a female (length: 80-90mm) can release 10-15 million eggs. The percentage of mature oysters was found to be high during April-May and August-September in Tuticorin indicating this is the peak spawning time (Rajapandian and Rajan, 1987).

In *C.madrasensis* the eggs and sperms are discharged directly into the water, where fertilization and subsequent development take place. The sperms are discharged by contraction of muscles in the walls of the genital ducts. Sperms

are carried away by the outgoing water current and appear as a dense white stream emerging from the exhalent opening and quickly disperse in water. The female rhythmically ejects the eggs through the inhalant opening (Rajapandian and Rajan, 1987).

In fertilized eggs, the first cleavage occurs immediately after the formation of the second polar body within 45minutes. The velliger or straight-hinge stage is reached at the end of 20hrs and the larvae measure 66μm in length at that time. On day 7, the umbo stage is attained and the larvae measure 150μm. On day 13, the larvae grow to around 280μm and with the appearance of the eye spot, they attain the eyed stage. On 18th day, the pediveliger larvae with a functional foot measuring 330-350μm begin settling.

In *C. madrasensis,* growth of the spat is rapid during the first three months. A size of 38 mm is attained in 90 days registering a growth of 12.6 mm/month and at the end of one year an average size of 87 mm is recorded. Growth of oysters is related to food availability and environmental conditions, particularly temperature and salinity (Rajapandian and Rajan, 1987).

Condition of oyster denotes the degree of fatness of an oyster i.e. the extent to which the meat fills the shell cavity. The size of the soft body of an oyster undergoes changes and such changes are usually associated with spawning. This is accomplished by an increase in the size of the gonads during maturation followed by a considerable reduction after spawning. This is followed by a slow increase in meat weight. In temperate waters the increased level of glycogen has been associated with this phase. But in tropical waters, this phase of glycogen accumulation is not distinct since part of it is utilized for gonad development (Quayle, 1980). Oyster harvesting is done during the pre-spawning period when the meat condition is good. High condition factor of 90-120 has been observed before spawning,

indicating the plumpness of the meat. After spawning the meat is watery and thin (Rajapandian and Muthiah, 1987). Changes in the meat weight of oyster are important to oyster farming, since it determines the harvesting period. Thus the study of seasonal condition of the gonads and meat is essential for successful farming of the edible oyster.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. Collection

For this study, edible oysters ranging in size from 60-100mm were collected from natural oyster beds occurring in creeks (Plate 3.1.1) connected to Tuticorin Bay (8°48'N; 78°11'E). Samples were also collected from the oyster stock maintained in the edible oyster farm situated in Tuticorin Bay. Collected oysters were cleaned and maintained as broodstock in the shellfish hatchery at Tuticorin Research Centre of CMFRI.

3.2. Sea water supply system

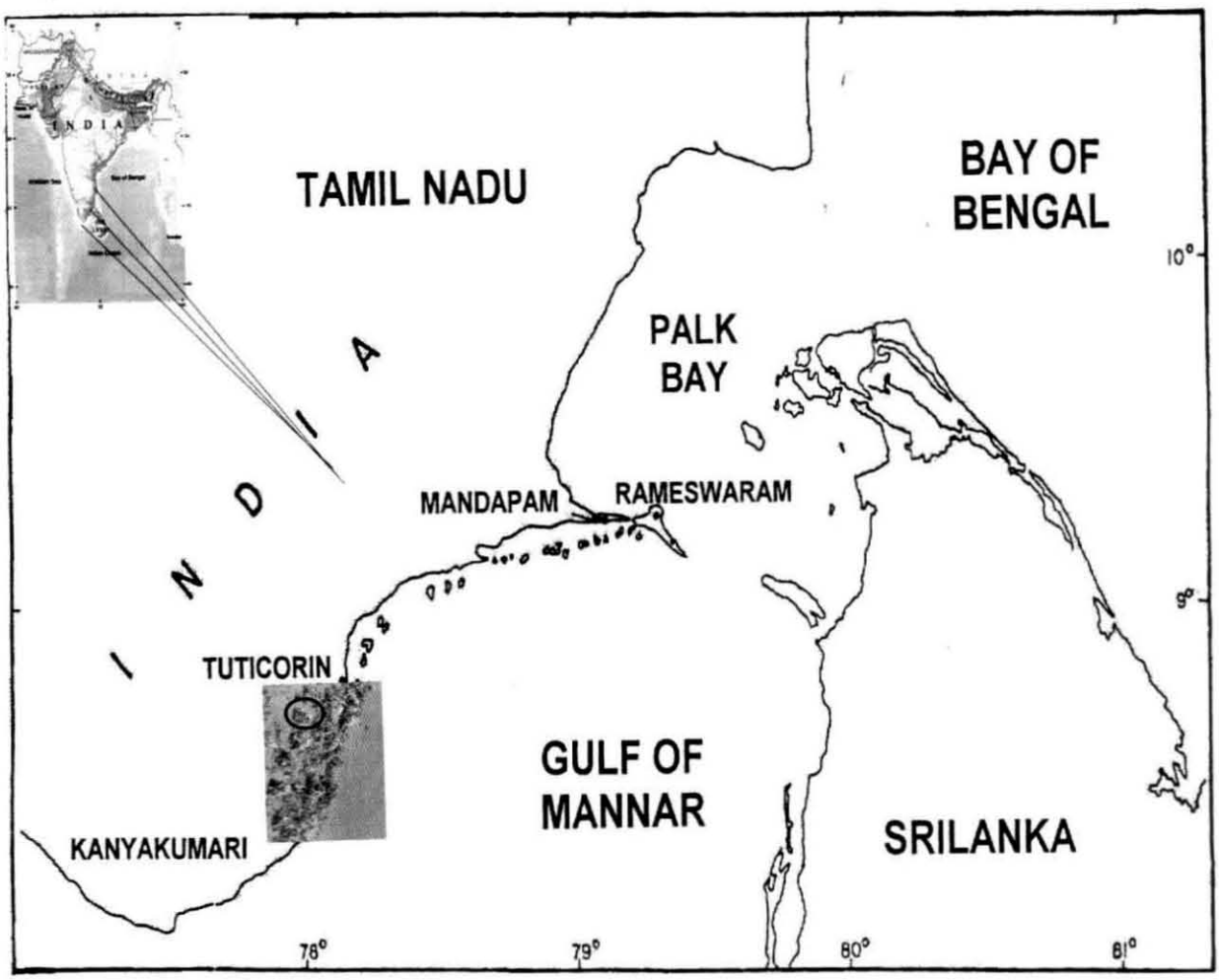
Oysters were kept in FRP tanks of size 75x50x50cm. The tanks were filled with filtered seawater. For supply of filtered seawater, raw seawater was pumped from Tuticorin bay to a filtration unit. Pumped water was passed through a sedimentation tank and filter bed, consisting of layers of gravel and fine river sand. The water thus filtered, was stored in a sump. From the sump, filtered seawater was pumped to an over-head tank. Through a pipeline, filtered seawater was supplied to the hatchery with adjustable gate-valves. Seawater for broodstock oysters was changed daily. In larval rearing, water was changed completely on alternate days. The larvae were reared in 50 litre FRP beakers; filtered seawater was supplied through a cotton pad tied at the delivery end of the PVC pipes. 5 H.P air compressors provided gentle aeration.

3.3.Feed culture

3.3.1. For Broodstock

For maintaining the broodstock and rearing of the larvae, microalgae were provided as feed. For broodstock, mixed algae consisting of *Chaetoceros calcitrans, Skeletonema* sp., and *Nitzchia* sp., were cultured in outdoor tanks using NPK, urea and sodium silicate (Gopinathan, 1996). Mixed algae having 1 to 1.5 million cells/ml were fed at a rate of 3.5 l per oyster per day. After

Plate 3.1.1.



Edible oyster bed exposed during low tide

ten days of feeding, a sample of oysters was cleaned to observe gonadal maturity stages by the smear method.

3.3.2. For larvae

The dinoflagellate, *Isochrysis galbana was* used as a food for the larvae. For culturing *I.galbana*, a serial dilution culture technique was employed. Walne's medium was used for mass culturing the phytoflagellate (Gopinathan, 1996). A fully-grown culture of *I. galbana* on 5-6 day with a 0.6million cells/ml was used for feeding the larvae.

3.4.Experiments

Detailed investigations were made on the induction for producing triploids. For assessing successful triploidy induction, metaphase chromosome spreads were prepared. The seed produced were reared in the farm. The growth of meiotic I and meiotic II triploids and diploid oyster seed were studied. Electrophoretic banding patterns of the three groups were also recorded. The biochemical constituents of triploid and diploid oysters were analyzed.

3.5. Statistical Analysis

Analysis to correlate the morphological and biochemical parameters of triploid and diploid oysters were performed using Systat-7.0.1 software. For "t" tests MS Excel programme of Windows 2000 was used.

CHAPTER 4

TRIPLOIDY INDUCTION

4.1. Introduction

In bivalves, triploidy can be induced either by inhibiting the polar body I or II, or by crossing tetraploids with diploids. Physical agents (pressure, thermal and cold shocks) and chemical treatments have been reported for induction of triploidy in bivalves (Beaumont and Fairbrother, 1991). Pressure treatments at 6000–8000 psi administered 10 minutes after fertilization for 10 minutes duration produced 57% triploids in *Crassostrea gigas* (Chaiton and Allen, 1985). Quillet and Panelay (1986) attempted thermal shock for triploidy induction in *C. gigas*. A high percentage (70–98%) of triploids was produced at temperatures between 28°C-35°C with the highest yield at 32°C (Yamamoto and Sugawara, 1988). In some, two agents (Yamamoto *et al.*, 1988; Beaumont and Kelly, 1989; Gosling and Nolan, 1989) or three agents (Wada *et al.*, 1989) were employed for inducement of triploidy.

A number of chemicals like Cytochalasin B (CB), Cytochalasin D and 6-Dimethylaminopurine (6-DMAP), Caffeine and Colchicine have been reported to be useful for induction of triploidy. Downing and Allen (1987), after comparing different chemicals used for inducement of triploidy, suggested that CB is more effective. Using CB, triploidy was produced in the oysters *Crassostrea virginica* (Stanley *et al.*, 1981) and *C.gigas* (Quillet and Panelay, 1986), in the clams *Mya arenaria* (Allen *et al.*, 1982) and *Tapes semidecussatus* (Beaumont and Contaris, 1988), and in the scallop *Argopecten irradians* (Tabarini, 1984), *Pecten maximus* (Beaumont, 1986), *Chlamys mobilis* (Komaru *et al.*, 1988) and *C. varia* (Baron *et al.*, 1989) and in the pearl oyster, *Pinctada fucata martensii* (Uchimura *et al.*, 1989). Among the six triploidy inducers (CB, heat, calcium, caffeine, combined calcium & heat and combined caffeine & heat), CB was considered the most effective agents in production of viable triploids (Scarpa *et al.*, 1994). The optimal treatment, duration and concentration of CB for triploidy

induction were determined for the Pacific oyster by Downing and Allen, (1987) and Allen *et al.* (1989), for Eastern oysters by Stanley *et al.* (1981); Barber *et al.* (1992) and Supan *et al.* (2000) and for Sydney rock oysters by Nell *et al.* (1996) and Hand *et al.* (2004). The application of CB was found to be successful for triploidy induction in the European flat oysters also by Gendreau and Grizel, (1990) and Hawkins *et al.* (1994). CB, being carcinogenic, is considered toxic and is potentionally harmful to the operators (Chew, 1994; Guo and Allen, 1994). As an alternative to CB for triploidy induction in Pacific oysters, 6 dimethylaminopurine (6-DMAP) was recommended (Desrosiers *et al.*, 1993; Gerard *et al.*, 1994)

Ever since the first successful triploidy attempt by Stanley *et al.* (1981) in oysters, triploidy has been induced in a number of bivalves like *C. gigas*, *C. virginica* and *Saccostrea glomerata* and *Ostrea edulis* (Nell, 2002). Commercial production of triploid oysters began in 1985 along the coast of America. Since physical methods are not dependable and chemical methods are costly, the use of tetraploid males to fertilize eggs from diploids to produce triploids has been developed recently (Nell, 2002). Subsequently, some hatcheries adopted the method of crossing tetraploid males to fertilize diploid females for producing 100% triploidy instead of chemical or physical inducement methods (Chew, 2000).

Advantages of triploids are increases in growth rate, higher dry meat weight and higher condition index values and disease resistance (Nell *et al.*, 1994), larger adductor muscles in scallops and increased survival rate in the Chinese pearl oyster *P. martensii* (Allen, 1998). In Pacific oysters (Allen and Downing, 1986) and in the Sydney rock oyster (Nell *et al.*, 1994), sterile triploids with higher meat condition overcome the problem of unmarketability of diploids due to sexual maturation, in summer and autumn.

Triploidy in oysters can be produced by suppressing meiosis I (M I) or II (M II) (Beaumont and Fairbrother, 1991). Suppressing meiosis II was reported to yield higher percentages of triploids and better larval survival (Hand *et al.*, 1999), whereas suppression of meiosis I results in a high proportion of aneuploids and hence higher mortality (Guo *et al.*, 1992). Stanley *et al.* (1984) reported faster growth of M I triploids over M II triploids.

In India, the commercially important edible oyster *Crassostrea madrasensis* is distributed all along the east and west coasts. Considering its nutritive value, Central Marine Fisheries Research Institute evolved suitable methods for farming the edible oyster (Nayar, 1987), which were successfully adopted by fishermen (Appukuttan, 2001). By establishing a shellfish hatchery, the CMFRI has developed techniques for mass production of edible oyster seed through hatchery systems (Nayar *et al.*, 1987). So far triploidy induction in the edible oyster *C. madrasensis* has not been tried. Hence, attempts were made in this study and various methods of triploid induction were applied to this species.

4.2.Materials and method

4.2.1.Collection and conditioning of oyster

Oysters of length 60–90 mm were collected from natural oyster beds occurring in the creeks adjacent to the Tuticorin Bay and also from the oyster farm maintained by the Tuticorin Centre of CMFRI at Tuticorin Bay. Samples of 10 oysters were opened for gonadal maturity studies. If the gonad was ripe, the oysters were induced to spawn. If the gonadal condition was in maturing stage, oysters were kept in the conditioning room where they were fed intensively with a mixed algal culture at 22 $\pm$ 1°C (Nayar *et al.*, 1987). After 10–15 days, on assessing gonadal condition, oysters were then used for induced spawning experiments.

4.2.2. Induced spawning

Ripe oysters were subjected to thermal stimulation at 32°C as attempted by Nayar *et al.* (1987). At the time of spawning, female and male oysters were taken out and kept separately in 5 l glass beakers. On completion of spawning, oysters were removed from the beaker. If spawning did not occur, a stripping method was attempted. The gonadal materials stripped from male and female oysters were kept separately in 5 l glass beakers. The released gonadal materials were passed through 100µm sieve so as to remove dirt and pieces of meat.

The number of eggs in the glass beaker was counted using a Sedgewick rafter cell counter (Nayar *et al.*, 1984). After stirring well 1 ml sub sample was pipetted into a counting chamber for enumeration. An average of three sub samples was taken for estimation of total egg number. An adequate quantity of sperm was added to the beaker containing the eggs to facilitate fertilization.

4.2.3.Kinetics of polar body

Since the efficiency of triploid induction depends on the synchrony of meiosis in eggs (Downing and Allen, 1987), the time of initiation and duration of treatment is very important. The ideal time for physical or chemical treatment of freshly fertilized eggs for blocking extrusion of second polar body is after the extrusion of the first polar body at the metaphase II stage. However, since developmental events between eggs are not well synchronized in oysters, there will be some overlap in the process of extrusion of the first and second polar bodies. Nell *et al.* (1996) suggested that the treatment should commence at the time when 50% of the first polar body has been extruded. Therefore, the kinetics of polar body extrusion was monitored by viewing the freshly fertilized eggs under light microscope. A sample of the fertilized gametes was taken and the biological development of the

freshly fertilized eggs were visualized under a microscope to monitor the kinetics of the polar body extrusion. As the polar-body extrusion is directly related to temperature, the time of extrusion of 50% polar body at 22 ± 1, 28 ±1 and 31±1⁰ C were determined.

4.2.4. Induction of second meiotic triploidy

Trials were carried out for induction of triploidy using physical agents like heat shock and cold shock as well as the chemical agents like Cytochalasin B and 6 - Dimethyl aminopurine. Different trials were carried out at varying dosages, initiation time and duration of treatment to determine the optimum treatment conditions for maximum triploid yield. For this fertilized eggs were collected in a 20 µm sieve and were subjected to heat, cold or chemical treatments as shown in Fig. 4.2.4.1 for induction of triploidy.

4.2.4.1. Physical shock

Cold shock treatment

Fertilized eggs were exposed separately to low temperatures of 5⁰C and 12⁰C. In each, the duration of treatment was 10 and 20 minutes. The treatment was given 17 minutes after fertilization. Fertilized eggs were collected in 20µm sieve, transferred to 3-l beaker containing seawater of low temperature (5⁰C and 12⁰C) maintained by adding ice.

Heat shock treatment

Fertilized eggs retained in a 20µm sieve were heat treated by immersing them in 35°C water for 5 min. Likewise treatments were given at temperatures 37 and 39⁰C. Treatment was given 17min after fertilization. Triplicates were maintained of each treatment and each duration of treatment. The required temperature was maintained by adding heated seawater.

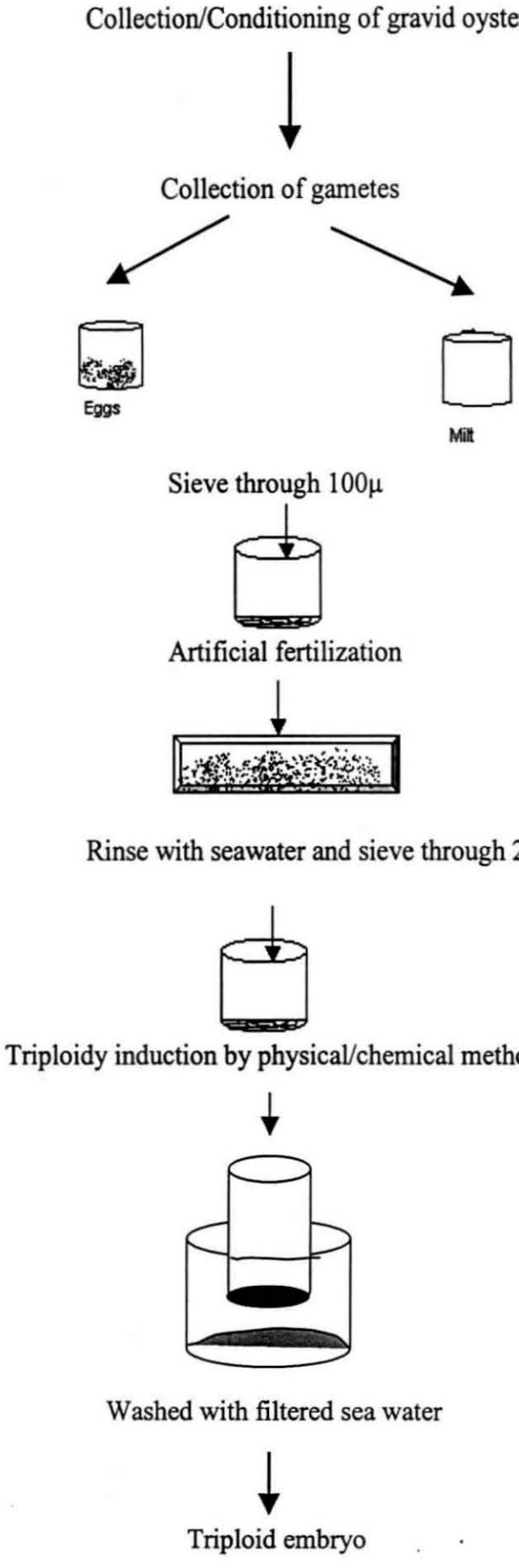


Fig. 4.2.4.1. Scheme for triploid induction in Edible oyster

4.2.4.2. Chemical treatment

Cytochalasin B (CB)

Experiments with various concentrations of CB were carried out to determine the dose-dependent inducibility of triploid. For this, crystalline CB (Sigma chemicals) was dissolved in dimethylsulphoxide (DMSO) and stored as 1ml aliquots at 0°C (Allen *et al.*, 1989). Three concentrations of CB i.e. 0.05, 0.10 and 0.15 mg/l were tried. Treatment was for 3 min, at 26°C (in air-conditioned room). When 50% of the fertilized eggs kept in 1-lit beaker extruded the I polar body, they were incubated with CB of different concentrations. After 3 min exposure, eggs were rinsed in DMSO (0.01mg/l) to remove any residual CB (Beaumont and Kelly, 1989). The control group was treated in the same way as the CB groups but in a solution of 0.01% DMSO but without CB for 3 min, followed by a washing in 0.01% DMSO for 1 min.

6–Dimethylaminopurine (6 –DMAP)

Fertilized eggs were incubated with 6-DMAP at different concentrations, viz., 0 (Control), 50, 100, 150 and 200µM (0, 8.15, 16.3, 24.5,32.6 mg/l). Three durations of exposure viz., 5, 8 and 10 minutes were applied for each dose. Treatment was carried out at room temperature (29°C). For this fertilized eggs collected in 20µ mesh were divided into 16 aliquots and each was subjected to a particular treatment. The time of initiation of treatment was when 50% of the fertilized egg extruded the I polar body.

4.2.5. Larval rearing

Fertilized eggs treated for triploidy induction were transferred to 40 l plastic troughs having filtered seawater and the larvae were reared at a density of 5 larvae/ml. For each treatment, triplicates with controls were maintained. The 'D' shape larvae were fed with *I. galbana* at the rate of 5000 cells/larvae/day, a concentration increased to 8,000 and 10,000 cells/larvae/day in the pediveliger and spat stages respectively as

practiced by Nayar *et al.* (1987), for the larval rearing of *C. madrasensis*. Mass culture of *I. galbana* was utilized as feed. Half of the filtered seawater was changed each day and a complete change affected on alternate days. Water temperature ranged from 29 to 31°C, salinity varied from 31 to 33 ppt and pH from 8.1-8.2, during the course of the experiments. Once in three days the contents of each trough were collected using a 50µm sieve and transferred to 3 l beaker. An aliquot of 1 ml was pipetted into a counting chamber to estimate the surviving number of larvae. Estimation was carried out for triplicates and larval survival rate determined per treatment.

4.2.6. Triploidy determination

Ploidy determination was carried out by counting the number of chromosomes in egg nuclei immediately after treatment and also for the 'D' shaped larvae on the 3rd day, 48 hrs after fertilization (Allen and Bushek, 1992). Just after treatment, 5 ml of sample was taken from the sieve and treated with 0.02% of colchicine for 1 hour. For the hypotonic treatment, the sample was transferred to 10 ml of 50% seawater for 20 min. Then after pipetting out seawater a few drops of cold Carnoy's solution (3:1, absolute methanol: glacial acetic acid) was added and the supernatant fluid was removed after 5 min. This process was repeated three times at an interval of 10, 15 and 20 min. A few drops of 50% acetic acid (refrigerated) were then added and mixed thoroughly using a Pasteur pipette. This suspension was then dropped on to a warm glass slide (50°C) placed on a slide warmer from a height of 30 cm. The slides were air dried and stained in Giemsa in phosphate buffer (pH 6.8) for 20 min. For preparation of metaphase plates for 'D' stage larvae, 0.02% colchicine (for 1 hr) treated larvae were crushed by sandwiching them in between two glass slides. After hypotonic treatment, the same procedure was followed for preparation of metaphase plates. For preparation of chromosome spreads from adult oysters, the oysters were fed for

6 hrs with *I.galbana* mixed with 0.05% colchicine solution. Gill tissue was dissected out and hypotonized in cold 0.9% Sodium citrate for 30 min, fixed in several changes of freshly made Carnoy's fixative and stored in absolute methanol at 4°C until used. During slide preparation, tissue was removed from methanol, dabbed with tissue paper to remove excess alcohol, minced in 50% glacial acetic acid and allowed to stand for 10 min and the same above procedure was followed. Chromosomes were counted under microscope. Based on the chromosome numbers the ploidy conditions (18 – 21 diploid, 25 -34 triploid (Plate 4.2.6.1) and 35 – 44 tetraploid)) were assessed (Yamamoto *et al.*, 1988).

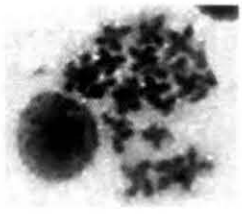
4.2.7. Induction of first meiotic triploidy

For inducement of I meiotic triploids, treatments were given to zygotes 8 min after fertilization. The treatments which were found to be optimum for induction of II meiotic triploid (cold 5°C, heat 37°C, 100μM 6-DMAP, 0.05mg/l CB and 0.01 mg/l DMSO) were only employed for induction of I meiotic triploidy. Other procedures were the same as adopted for the II meiotic triploid.

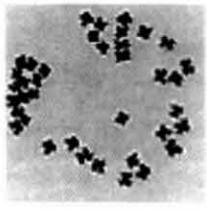
4.3.Results

The kinetics of polar body I and II extrusion in *C. madrasensis* at three different temperatures are given in Figure.4.3.1. The 1st polar body extruded at 8 min after fertilization at 31°C an interval increased to 10 and 12 min at 29 and 23°C, respectively. 50% of 1st polar body formation was observed at 15, 16 and 18 minutes after fertilization at 31, 29 and 23°C, respectively. The occurrence of 2nd polar body varied from 21 min (at 31°C) to 23 min (23°C). 50% of 2nd polar body had formed at 28, 32 and 36 minutes at 31, 29 and 23° C respectively. Cleavage occurred at 34min (at 31°C), 38min (at 29°C) and 43 min (23 °C) respectively.

Plate. 4.2.6.1



2n=20



3n=30

Metaphase spread of *C.madrasensis*

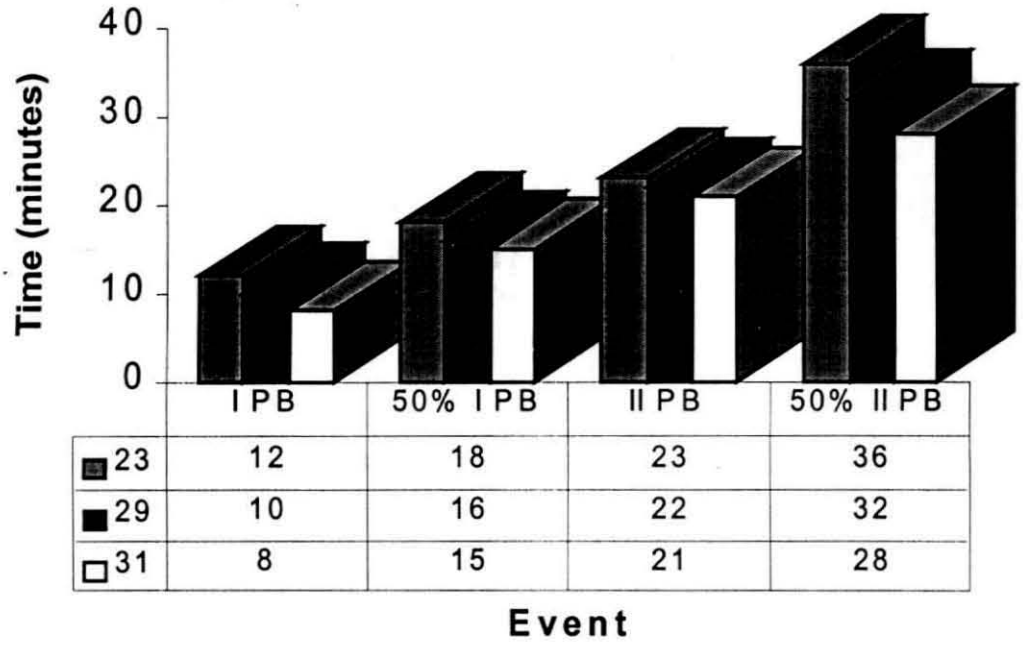


Fig. 4.3.1. Extrusion of polar body at different temperatures (in °C)

Triploid induction efficiency of cold shocks treatments are presented in Table 4.3.1. Cold shock at 5°C for 10 minutes resulted in 42.25% triploidy; by prolonging the exposure time to 20 minutes, the percentage was 40.92. On the third day, the percentage was 33.33 and 30.33 for 10 and 20 min duration, respectively. Cold shock at 12°C for 10 minutes duration yielded 35.29% triploids as against 36.6% for 20 minute exposure. However in 'D' stage the percentage triploidy was similar for all treatments (32%).

Triploid percentage at the embryonic stage, resulting from heat shock are presented in Table 4.3.2. High triploid percentages of 42 ± 1.24 and 41.85 ± 2.03% were obtained from heat shock at 37°C for 5 and 10 min duration, respectively. At 35°C and 39°C, the percentages of triploidy were 41.18 ± 0.78 and 40.75 ± 0.83, respectively, for the 10-minute duration. In the 'D' stage the percentage of triploidy was 38.82 ± 1.55 and 39.13 ± 1.53 % for both 10 and 5 min treatment at 37°C. For both the durations of treatment at 39°C the percentage was 38. Lowest percentage of 30.8 to 34.4 was observed for the heat shock at 35°C for 5 and 10 min, respectively.

Among the three CB concentrations (0.05, 0.10 and 0.15 mg/l), the highest percentage of triploid embryo was obtained from 0.05 mg/l CB. The values for the embryo and 'D'shape larvae were 41.8 ± 1.04 % and 40 ± 1.2%, respectively. In the 0.10 and 0.15 mg/l, groups treated the percentage of triploids varied from 36.18 ± 0.56 to 39.50 ± 0.72 (Table 4.3.3). In control all were diploids.

The percentage of triploidy induced by 6-DMAP treatments are given in Table 4.3.4. Among the different concentrations tried, 50,100 and 150µM concentrations applied for 8 minutes resulted in high triploid yield exceeding 60% while moderate level of triploidy (40.75 to 58.75%) was obtained from 200µM. The

Table 4.3.1. **Relative efficiency of different cold shock treatments for induction of triploidy in oysters***

Temperature	Percentage of triploids (First day larvae)		Percentage of triploids (D shaped larvae)	
	Duration of treatment		Duration of treatment	
	10'	20'	10'	20'
5°C	42.25±1.67	40.92±1.67	33.33±1.21	30.33±0.63
12°C	35.29±1.07	36.66±0.82	32.48±0.75	32.00±1.12

*Values are means ± SE (number of replicates =3)

Table 4.3.2. **Relative efficiency of various temperatures and duration of heat shock for induction of triploidy in oysters***

Temperature	Percentage of triploids (First day larvae)		Percentage of triploids (D shaped larvae)	
	Duration of treatment		Duration of treatment	
	5'	10'	5'	10'
35°C	40.44±2.3	41.18±0.78	30.84±0.65	34.44±0.66
37°C	42.00±1.24	41.85±2.03	39.13±1.53	38.82±1.55
39°C	41.38±0.38	40.75±0.83	38.18±0.5	38.00±0.62

*Values are means ± SE (number of replicates =3)

Table 4.3.3. **Relative efficiency of different concentrations of CB at 26^0 C and 3 minutes duration***

Parameter	CB Concentration					
	0.05 mg/l		0.10 mg/l		0.15 mg/l	
	Larval	D shape	Larval	D shape	Larval	D shape
Triploid %	41.81±1.04	40.0±1.20	39.50±0.72	36.48±0.92	37.13±1.16	36.18±0.56

*Values are means ± SE (number of replicates =3)

Table 4.3.4. **Relative efficiency of different concentration and duration of 6- DMAP exposures for induction of triploidy in edible oyster ***

Duration / Concentration	Percentage of triploid					
	Larval stage			D stage		
	5min	8min	10min	5min	8min	10min
50µM	35.26±1.50	60.03±2.01	55.00±1.63	33.43±1.42	46.07±2.74	53.06±1.84
100µM	45.65±1.36	66.60±1.65	46.20±2.11	42.20±1.56	61.82±1.48	40.55±1.96
150µM	57.14±1.60	61.82±2.09	44.52±1.50	55.55±1.75	55.00±1.40	40.18±2.01
200µM	58.75±2.57	46.34±1.50	42.15±2.31	47.05±2.57	41.70±1.44	40.75±1.58

*Values are means ± SE (number of replicates =3)

concentration of 100μM with an exposure of 8 minutes was found to be optimal yielding a high percentage for both embryonic (66.6%) and 'D'shape larvae (61.8 %) (Table 4.3.4).In control all were diploids.

A comparison of the relative efficiency for optimum outcome of physical and chemical treatments, are presented in Table 4.3.5. The percentages of 2nd meiotic triploids were 41.8 in CB treatment and 66.6 in 6-DMAP treatment for the embryos. For the 'D' shape larvae also 6-DMAP gave the highest rates 61.82 followed by cold treatment (42.25%). For heat treatment 40.9 and 39.1% were observed among the embryos and 'D' shape larvae, respectively. In cold treatment, 42.2 and 33.3% were attained for embryos and 'D' stage, respectively. In the DMSO treatment, the percentage was 3.25 – 5.1. Mortality varied from 13.2% in heat-treatment to a maximum of 17% in 6–DMAP treatment whereas in the control the mortality was 10.2 % (Table 4.3.5). Compared to 2nd meiotic triploids, 1st meiotic triploids produced by 6-DMAP at a concentration of 100μM for 8 minutes gave 60.57% triploidy with 13.70% mortality (Table 4.3.6.).

4.4. Discussion

The kinetics of polar body extrusion at three temperatures indicated that the timings were directly related to the prevailing temperature (29°C). The 50% of I polar body extrusion occurred between 15 to 18 min which conforms with the observation of polar body extrusion in 15 min after fertilization in *C. gigas* by Gerald *et al.* (1994).

Since Ist polar body extrusion started 10 min after fertilization, treatments were given 8 min after fertilization for arresting the polar body I to produce Ist meiotic triploids. And for production of II meiotic triploids treatments were applied 17 min after fertilization. In *C. gigas*, temperature treatment at 5-30 min

Table 4.3.5. **Relative efficiency of different treatments for induction of II meiotic triploids in oyster**

Treatment	Triploid%		Mortality % (5^{th} day)
	Larval	D stage	
6-DMAP (100 μM)	66.60	61.82	17.00
Cold (5^0C)	42.25	33.33	16.08
Heat (37^0C)	40.92	39.13	13.20
CB (0.05mg/l)	41.81	40.0	14.37
DMSO (0.01mg/I)	3.25	5.10	7.81
Control	0	0	10.20

Table 4. 3. 6. **Comparison of I &II meiotic treatments for triploidy induction using 6-DMAP (100μM)***

Treatment	Triploid%		Mortality % (6^{th} day)
	Larval	D stage	
I Meiotic	60.57±0.82	58.42±0.52	13.70
II Meiotic	60.39±0.94	59.98±0.65	13.75
Control	0	0	11.18

*Values are means ± SE (n=3)

(Quillet and Panelay, 1986) and chemical treatment at 15 min (Desrosiers *et al.*, 1993) after fertilization were attempted.

With cold shock at 5°C for 10 and 20 minutes' duration, 42.3 and 40.9 % of triploids were obtained respectively. The optimum cold shock was 5°C for 10 minutes. However, the result was poor compared to 66.7 % obtained for *C.gigas* at 0°C for 10 minutes (Yamamoto *et al.*, 1988) and 85.3% in *Mytilus edulis* at 1°C for 10 minutes (Yamamoto and Sugawara, 1988). The higher percentages obtained in these studies may be attributed to the lower treatment temperatures (0-1°C) used than 5°C attempted in this study. ANOVA at larval stage gave significant differences among triploid percentages between the two treatment at two different duration (Table 4.3.7), but no significant difference was observed at the 'D' stage (P>0.1).

Triploid percentage realized from heat shock at 37°C for 5 and 10 min were 42 % and 39 %, respectively. Analysis of variance showed that the differential percentages of triploids among 'D' stage larvae produced at temperatures 35, 37 and 39°C were significant (Table 4.3.8), but the differences during larval stage were not (P>0.1). Quillet and Panelay (1986) reported triploid levels of 25–45% after heat shock at 35 and 38°C in *C. gigas*. The wide range of triploidy rates may result from treatment being given 10-40 minutes after fertilization.

A concentration of 0.05 mg/l and a duration of 3 minutes were found to be the best parameter for inducing triploidy percentage (40.0 - 41.8%). Analysis of variance indicated that triploidy percentage in 'D' stage larvae from 0.05, 0.10 and 0.15 mg/l treatments was significantly different ($F = 19.401$; df 2; $p<0.05$) (Table: 4.3.9). No significant difference was seen at the larval stage (P>0.1) Whereas in *Mytilus galloprovincialis*, 80% triploidy was observed after 15 min exposure at 1 mg/l CB (Scarpa *et.al.*,1994), while only 67% was reported for *M. edulis* (Beaumont

Table 4.3.7. **ANOVA on percentage of triploidy produced by cold shock (Larval stage)**

Source	Sum- of – Squares	df	Mean- Square	F-ratio	P
Treatment	535.469	1	535.469	119.647	0.000
Duration	116.439	1	116.439	26.017	0.001
Treatment& Duration	173.280	1	173.280	38.718	0.000
Error	35.803	8	4.475		

(Also refer Table 4.3.1)

Table 4.3.8. **ANOVA on percentage of triploidy produced by heat shock (D stage)**

	Sum- of – Squares	df	Mean- Square	F-ratio	P
Treatment	141.236	2	70.618	22.379	0.000
Duration	4.836	1	4.836	1.533	0.239
Treatment& Duration	14.797	2	7.398	2.345	0.138
Error	37.867	12	3.156		

(Also refer Table 4.3.2)

Table 4.3.9. **ANOVA on percentage of triploidy produced by CB (Dstage)**

Source	Sum- of– Squares	df	Mean- Square	F- ratio	P
Treatment	88.316	2	44.158	19.401	0.002
Error	13.656	6	2.276		

(Also refer Table 4.3.3)

and Kelly, 1989). The intermediate rates of triploids in this study may be attributed to the low duration of treatment.

Of the different concentrations of 6-DMAP applied (50-200μM), 100μM applied for 8 min duration was optimum and yielded more triploids than did 5 either 10 min treatment intervals. As concentration increased to 200μM, a shorter duration 5 min treatment produced more triploids than did the other two durations. Analysis of variance of the percentages of triploid larvae (Table 4.3.10) and "D" stage larvae (Table 4.3.11) showed that the differences among treatments at various concentrations and durations of 6-DMAP were significant. At high concentrations of 6-DMAP, shorter duration of exposure can be effective as Desrosiers *et al.* (1993) observed, higher concentrations and the longer duration of treatment with 6–DMAP increased abnormalities in *C. gigas*.

Among the various physical and chemical treatments tried for producing II meiotic triploids, 6-DMAP yielded the highest percentage (66.6%). Similarly, Desrosiers *et al.* (1993) obtained high percentage of 90–95% in 6–DMAP treated eggs of *C. gigas* when compared to 57% for pressure shock (Chaiton and Allen, 1985), 83% for heat shock (Yamamoto *et al.,* 1988) and 90% for cytochalasin B treatment (Downing and Allen, 1987). The DMSO alone has a mild inhibitory effect on the extrusion of polar bodies. Similarly Desrosiers *et al.* (1993) indicated slight inhibitory effect on the polar body extrusion in the giant scallop *Placopecten magellanicus*.

Among treatments, the most efficient (61.82%) was 6-DMAP. The three other treatments produced an average of 39.13% (heat), 41.81% (CB), and 33.33 % (cold) respectively. The results indicate that of all treatments used in the present study, 6-DMAP is ideal for induction of triploidy in *C.madrasensis*. In *C.gigas* also, high yield of triploids were observed after 6-DMAP treatment (Desrosiers *et al.,* 1993). Though heat shock will produce triploids,

Table. 4.3. 10. **ANOVA on percentage of triploidy produced by 6-DMAP (Larval)**

Source	Sum- of - Squares	df	Mean- Square	F- ratio	P
Treatment	166.142	3	55.381	5.331	0.006
Duration	931.118	2	465.559	44.813	0.000
Treatment& Duration	1880.350	6	313.392	30.166	0.000
Error	249.336	24	10.389		

(Also refer Table 4.3.4)

Table. 4.3.11. **ANOVA on percentage of triploidy produced by 6 - DMAP (D-stage)**

Source	Sum- of - Squares	df	Mean- Square	F-ratio	P
Treatment	289.381	3	96.460	9.236	0.000
Duration	397.299	2	198.650	19.021	0.000
Treatment& Duration	1562.124	6	260.354	24.929	0.000
Error	250.649	24	10.444		

(Also refer Table 4.3.4)

Nell (2002) stated that physical methods are not always reliable. Chemical methods also do not guarantee 100% triploidy. Moreover, the chemicals are costlier and use of CB is potentially dangerous (Nell, 2002). Chew (1994) suggested that these problems could be overcome, if triploids were produced by mating tetraploids with normal diploids. The majority of triploid Pacific oysters in France and US are produced by fertilizing tetraploid males to diploid females (Nell, 2002). Therefore, future efforts will be directed at developing techniques for production of tetraploid in *C.madrasensis* with a view to produce triploid oysters by crossing tetraploid with diploid individuals.

CHAPTER 5

ALLOZYME ELECTROPHORESIS

5.1.Introduction

Genetic studies relevant to hatchery production of bivalves have been carried out routinely over the last 20 years using genetic markers, such as allozymes. Different electrophoretic forms of an enzyme, which are the products of alternative alleles segregating at a locus within a species, are called allozymes, while isozymes are alternative forms of an enzyme produced by different loci (Ferguson, 1980). Thus each isozyme can potentially exist in a number of allozymic states (Ferguson, 1980). Allozyme electrophoresis enables detection of different alleles at enzyme coding loci that produce proteins, which migrate different distance through a supporting gel (Beaumont, 2000). Furthermore, the methodology is relatively simple, requires only inexpensive equipment and can readily be used to process large numbers of individuals. Allozyme electrophoresis has three additional strengths – a well developed theoretical basis, broadly accepted and standardized approaches for statistical analysis and hypothesis testing databases that provide an invaluable background against which to evaluate new data (Shaklee and Benzen, 1998).

Allozymes are mostly primary products of transcriptionally active genes and it is assumed that a specific enzyme profile is the reflection of the genetic make up of a given species. They may be used to "fingerprint" a species/stock/individual considering all other variables as constant (Sarangi and Mandal, 1996). Allozymes offer a potentially powerful and reliable tool for resolving genetic relatedness/divergence questions by employing the degree of polymorphism of diverse alleles at different loci involved in translating specific enzymes and their varied multiple molecular forms. They have been used widely as molecular tags in genetic,

phylogenetic, taxonomic and evolutionary studies and in strain or type identification (Richardson *et al.*, 1986).

Electrophoretic separation of allozymes provide a visual presentation of the products of a single gene. The application of electrophoretic techniques on genetically controlled polymorphic enzymes can also be used to confirm gynogenesis and polyploidy (Ryman and Utter, 1986). Electrophoresis is particularly applicable for diagnosis of ploidy because it allows direct visualization of gene duplication at discrete structural gene loci (Allen *et al.*, 1982). It has also been applied effectively to a considerable array of studies to determine gene dosages in polyploid vertebrates (Balasano *et al.*, 1972).

In molluscs, the commonly used polymorphic isozymes are Phosphoglucomutase (PGM), Glucose phosphate isomerase (GPI), Leucine amino peptidase (LAP), Esterase (EST), Isocitrate dehydrogenase (IDH) and Glucose-6-phosphate dehydrogenase (G_6PDH) (Allen *et al.*, 1982; Hawkins *et al.*, 1994; Magoulas *et al.*, 2000). According to Allen *et al.* (1982) these enzymes were found to be sufficiently heterozygous for screening of diploids from polyploids. Examination and comparison of PGI phenotypes in heat shocked and control groups indicate exact conformance to theoretically expected staining ratios, enabling separation of diploids and triploids in experimental groups (Crozier and Mofett, 1989). According to Balasano *et al.* (1972) it is also possible to determine the frequency of triploidy by screening albumin phenotypes in natural populations. Beaumont and Kelly (1989) point out that triploid mussels can serve as a useful research tool to address current genetic phenomena such as heterozygosity with growth correlations and heterozygote deficiencies in marine bivalves. In the induction of triploidy by targeting meiosis I, the homologous chromosomes that normally separate from each other at this stage of cell division are prevented from doing so, and thus, all of the heterozygosity of the female

phylogenetic, taxonomic and evolutionary studies and in strain or type identification (Richardson *et al.*, 1986).

Electrophoretic separation of allozymes provide a visual presentation of the products of a single gene. The application of electrophoretic techniques on genetically controlled polymorphic enzymes can also be used to confirm gynogenesis and polyploidy (Ryman and Utter, 1986). Electrophoresis is particularly applicable for diagnosis of ploidy because it allows direct visualization of gene duplication at discrete structural gene loci (Allen *et al.*, 1982). It has also been applied effectively to a considerable array of studies to determine gene dosages in polyploid vertebrates (Balasano *et al.*, 1972).

In molluscs, the commonly used polymorphic isozymes are Phosphoglucomutase (PGM), Glucose phosphate isomerase (GPI), Leucine amino peptidase (LAP), Esterase (EST), Isocitrate dehydrogenase (IDH) and Glucose-6-phosphate dehydrogenase (G_6PDH) (Allen *et al.*, 1982; Hawkins *et al.*, 1994; Magoulas *et al.*, 2000). According to Allen *et al.* (1982) these enzymes were found to be sufficiently heterozygous for screening of diploids from polyploids. Examination and comparison of PGI phenotypes in heat shocked and control groups indicate exact conformance to theoretically expected staining ratios, enabling separation of diploids and triploids in experimental groups (Crozier and Mofett, 1989). According to Balasano *et al.* (1972) it is also possible to determine the frequency of triploidy by screening albumin phenotypes in natural populations. Beaumont and Kelly (1989) point out that triploid mussels can serve as a useful research tool to address current genetic phenomena such as heterozygosity with growth correlations and heterozygote deficiencies in marine bivalves. In the induction of triploidy by targeting meiosis I, the homologous chromosomes that normally separate from each other at this stage of cell division are prevented from doing so, and thus, all of the heterozygosity of the female

parent is retained in the egg. The addition of the male haploid chromosome set further increases heterozygosity beyond that of meiotic II induced triploids or diploids. It has been suggested by Stanley *et al.* (1984) that I meiotic triploids have higher heterozygosity levels than II meiotic triploids.

In the present work, allozyme profiles of three groups of oysters, a normal diploid (control), a triploid group produced by blocking polar body I (3nPBI) and a triploid group produced by blocking second polar body (3nPBII) with 6-DMAP have been resolved with the following objectives:

1. To evolve a suitable enzyme marker for triploid confirmation in the edible oyster; and
2. To compare the heterozygosity of I and II meiotic triploids over controls and to examine whether heterozygosity has got any positive correlation with the growth in triploid *C.madrasensis* oysters.

5.2. Materials and methods

5.2.1. Collection and transportation

Six month old hatchery produced edible oysters used in this study were from three groups, viz. A normal diploid (2n); triploid group produced by blocking polar body II with 6 DMAP (3nII) and a triploid group produced by blocking polar body I with 6-DMAP (3nI). Thirty oysters sampled randomly from each group, that were reared at Tuticorin, were transported to Kochi (340km away) in wet gunny bags avoiding mechanical damage. In the laboratory, they were transferred to aerated seawater (salinity: 32ppt; temperature: 29°C), and fed with mixed algae, (4,000 cells/oyster) on the next day.

5.2.2. Preparation of tissue extract

About 0.5 g adductor muscle was excised from each oyster for allozyme electrophoresis. The tissue was minced in the cold (4°C-10°C) and was homogenized using a homogenizer in

selected media. The homogenizing solutions consisted of distilled water, 0.2M sucrose-solution, or 0.05M Tris/HCl buffer (pH 7.0). The ratios of these media utilized for homogenizing the tissue were 1:1(w/v) 2:1 (w/v) and ½: 1 (w/v). Homogenates were taken in ice-cold eppendorf tubes and centrifuged at a speed of 10,000 rpm for 45 minutes at 4°C. The supernatant was transferred to another set of cold-labeled eppendorf tubes and centrifugation was repeated for another 30 minutes. The supernatant was stored at -85°C in New Brunswick Ultra low freezer until they were analyzed on the following day.

5.2.3. Electrophoresis

The supernatant was analyzed by vertical PAGE using a mini gel electrophoresis unit (Mighty small SE 250; Hoefer, USA). A stock solution (30%) of acrylamide and bisacrylamide was used. Three buffers were tried as given in Table 5.2.3.1.

Different gel percentages were tried to maximize resolution of enzyme bands. Composition of the gel buffer, including APS and TEMED in the gel mixture at different gel percentages are given in Table 5.2.3.2. The proportions tried for sample and loading buffer were 1:1 (v/v), 2:1 (v/v), 3:1(v/v), 4:1 (v/v) and 5:1(v/v). The loading buffer consisted of 1 ml of 0.5% bromophenol blue, 2ml of glycerol and 7ml of double distilled water. Samples were loaded at the cathodal end and electrophoretic apparatus was kept at 20°C in a refrigerator to minimize heat produced during the electrophoretic run. The run was stopped when the marker dye reached the anodal end. After the run was over, the gel was taken from the cassette and stained for specific isozymes.

Electrophoresis was carried out to assess allozyme variation for Esterase, Phosphoglucomutase, Glucose–6-Phosphate dehydrogenase, Glucose phosphate isomerase, and Superoxide dismutase (total 10 loci). These loci were selected because of high allelic variability reported in related species (Stanley *et al.*, 1984; Hawkins *et al.*, 2000; Wang *et al.*, 2002). A locus was considered

Table 5.2.3.1. **Electrophoresis buffers tried for activity and resolution of the different enzyme loci examined in *C.madrasensis***

Buffer	Electrode buffer	pH	Gel buffer	pH
	Components per 500ml		Components per 25ml	
Tris[1] Citrate Borate, pH 8.7 (TCB)	0.3M Borate 9.27g 60mM NaOH	8.1	0.5M Tris 1.51g (Adjust the pH with 2M citrate)	8.7
Tris Glycine, pH 8.2 (TG)	0.2M Glycine 7.51g (Adjust pH with 2M Tris)	8.2	1.8M Tris 5.45g (Adjust the pH with 3N HCl)	8.7
Tris EDTA[2] Borate, pH 8.4 (TEB)	150mM Tris 9.09g 3mM EDTA 0.56g 117mMBorate 3.62g	8.4	48mMTris 0.15g 1mM EDTA 0.009g 37mM Borate 0.06g	8.4

[1] Tris (hydroxy methyl) aminomethane

[2] Ethylene diamine tetra acetic acid

Table 5.2.3.2. **Composition of the gels used in the electrophoretic analysis in *C.madrasensis***

Ingredients	Gel composition					
	6.5%		8% gel		9%gel	
	For 15ml	For 25ml	For 15ml	For 25ml	For 15ml	For 25ml
Acrylamide[a]	3.25	5.41	4	6.6	4.5	7.5
Buffer	1.875	3.125	1.875	3.125	1.875	3.125
Double distilled water	1.25	3.95	1.25	2.08	1.125	1.875
N,N.N'N'-Tetramethylethylene diamine (TEMED)[b]	5	8.3	5	8.3	5	10
Ammonium per sulphate (APS)[c]	7.5	7.5	7.5	7.5	7.5	7.5

[a] 29.1 g Acrylamide weighed and added to 0.9g N,N'-Methylene bisacrylamide and dissolved in 50 ml double distilled water (DDW).

[b] microlitre.

[c] (0.1g/1ml) weighed and dissolved it in 1ml DDW. Taken 115µl of 10% APS and added to 7.5 ml for 15 ml and 118 µl for 25 ml.

as polymorphic only if its most common allele had a frequency not higher than 0.95. Details of staining recipes as followed by Shaw and Prasad (1970) are given in Appendix A. Agar overlay (2%) was used for PGM and GPI.

5.2.4.Statistical Analysis

Patterns of enzyme variation that were consistent with the subunit structure of the enzyme (when known) and simple models of mendelian inheritance were scored and recorded as zymograms (Shaklee, 1984).

In a stained gel when more than one staining zone appeared, the most anodal locus was designated as 1. The most common allele at each locus was designated as 100 and all other alleles at that locus were numbered according to their electrophoretic mobility relative to the 100 allele. Alleles with identical mobility at a particular locus were considered as homozygous. In oysters the two alleles are denoted as A and B. Homozygotes were represented as AA/BB for diploids and AAA/BBB for triploids as only one type of polypeptide will be produced. In the heterozygote, normally only two bands were found on the gel if the protein was a monomer and three bands were seen in dimers. In the diploid, heterozygotes were represented as AB and in triploids it was represented as AAB/ABB. From these assumed genotypes, the genotype frequencies and allelic frequencies were calculated for each locus in each sample. Heterozygosity for each locus (He) was calculated as

$$He = 1-\Sigma xi^{2}$$

where, xi is the frequency of the i th allele. The frequency of an allele in diploid was calculated by using the formula

$$\frac{2\,Ho + He}{2N}$$

Where, Ho = Number of homozygotes for that allele

He = Number of heterozygotes for that allele

N = Number of individuals examined

As 3 alleles were present in triploids the formula was modified as

$$\frac{3Ho + 2He' + He}{3N}$$

Where, Ho = Number of homozygotes for that allele

He' = Number of duplicated heterozygotes for that allele

He = Number of heterozygotes for that alleles

N = Number of individuals examined

It was possible to distinguish between a diploid genotype e.g. AB and a triploid genotype e.g. ABB/AAB on the basis of relative staining intensities of the component bands. The expected phenotypes of monomeric and dimeric enzymes in diploid and triploid staining patterns are given in Figure 5.2.4.1. Multiple locus heterozygosity was computed for each individual oyster as the number of studied loci that were heterozygous. A paired comparison't' test was conducted to compare single locus heterozygosity of the three groups; and a simple't' test for multiple locus heterozygosity was calculated.

5.3.Results

5.3.1.Standardization of the methodology

The details of the results of standardization made for individual allozymes were as described under each enzyme heading. Among the various homogenizing media tried, double distilled water of ½:1 (W/V) ratio gave the best results. Centrifugation of the tissue homogenate at a speed of 10,000 rpm for 45 minutes at 4°C was followed by spinning the supernatant again at the same speed and temperature for another 30 minutes. The polyacrylamide gel concentration standardized for PGM, GPI and SOD was 9%. An 8% gel was preferred for esterase and 6.5% gave better results for G_6PDH.

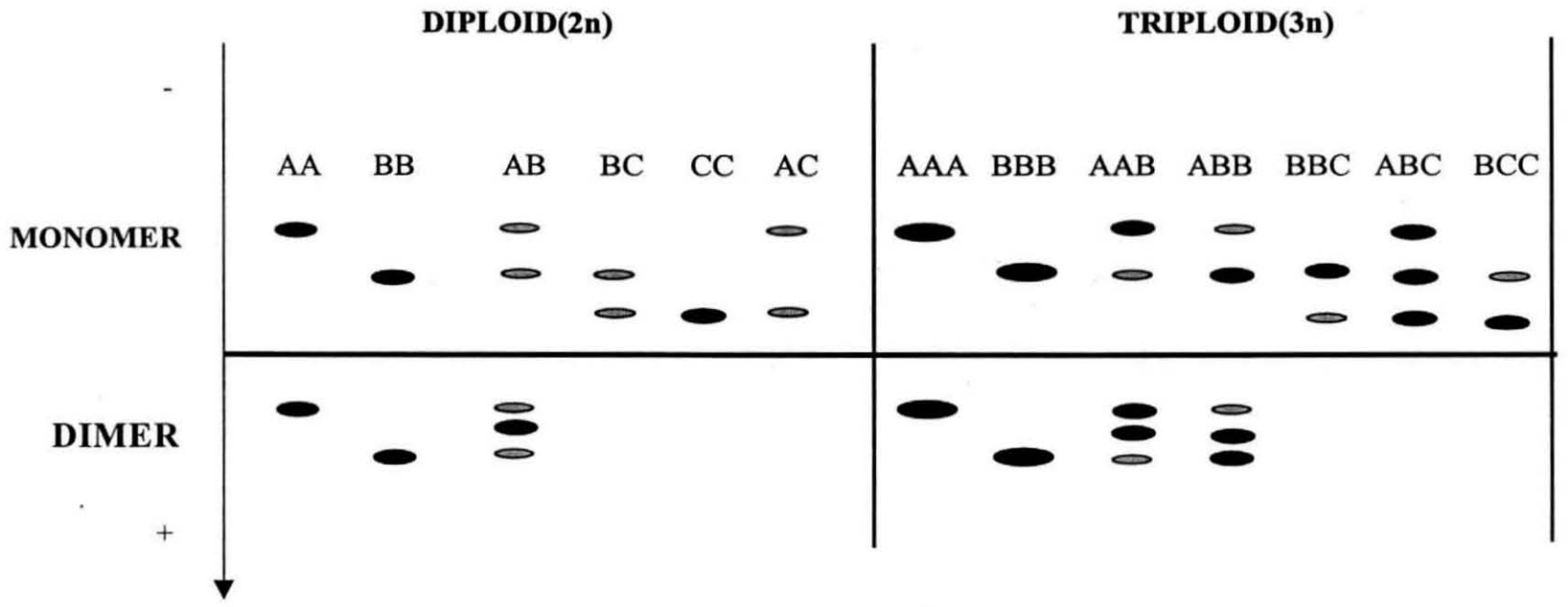


Fig. 5.2.4.1. Zymogram representation of observed phenotypes of monomeric and dimeric enzymes in diploid and triploid *Crassostrea madrasensis*

Homozygotes : AA/BB/CC/AAA/BBB

Heterozygotes : AB/BC/AC/AAB/ABB/BBC/ABC/BCC

Though tests were conducted to detect 6 enzymes, one enzyme leucine amino peptidase failed to show any activity with all the buffer systems tried. Of the five systems detected (total 10 loci), one locus for PGM, 3 loci for EST and 2 each for GPI, SOD and G_6PDH were scored. Numbers were assigned to loci sequentially in relation to the electrophoretic mobilities of the homomeric isozymes. Numbering began with "1" for the enzyme locus closest to the cathode and proceeded towards the anode. Banding patterns of all 10 loci were polymorphic. Details of results obtained under each enzyme system are given below.

Esterase (EST)

The zymogram of esterase showed a fast moving zone, an intermediate zone and a slow moving zone (Fig. 5.3.1.1) which apparently were determined by at least 3 gene loci (*Est-1**, *Est-2**, and *Est-3**). Intense esterase activity was observed in TCB (pH 8.7) buffer giving good resolution (Plate 5.3.1). 100,120 and 133 alleles were present at the *Est-1** locus, 100,116 and 125 alleles were present at the *Est-2** locus, and 100 and 109 alleles were present at the *Est –3** locus. Three alleles (A, B & C), with triallelic heterozygotes (ABC) were observed at the *Est-2** & *Est-3** locus of I meiotic triploids.

Superoxide dismutase (SOD)

SOD activity was detected as bleached areas on a dark background. TG buffer gave good resolution for this enzyme. For easy scoring the contrast between the bleached areas of enzyme activity and the dark background was increased by staining for a longer duration under indirect light. Two zones of activity were noted (Fig. 5.3.1.2.). Zone I of the control group was monomorphic with a single genotype (100) but exhibited polymorphism in triploids having four genotypes. The fast moving second zone exhibited variability that indicated polymorphism at the *SOD-2** locus (Plate 5.3.2).

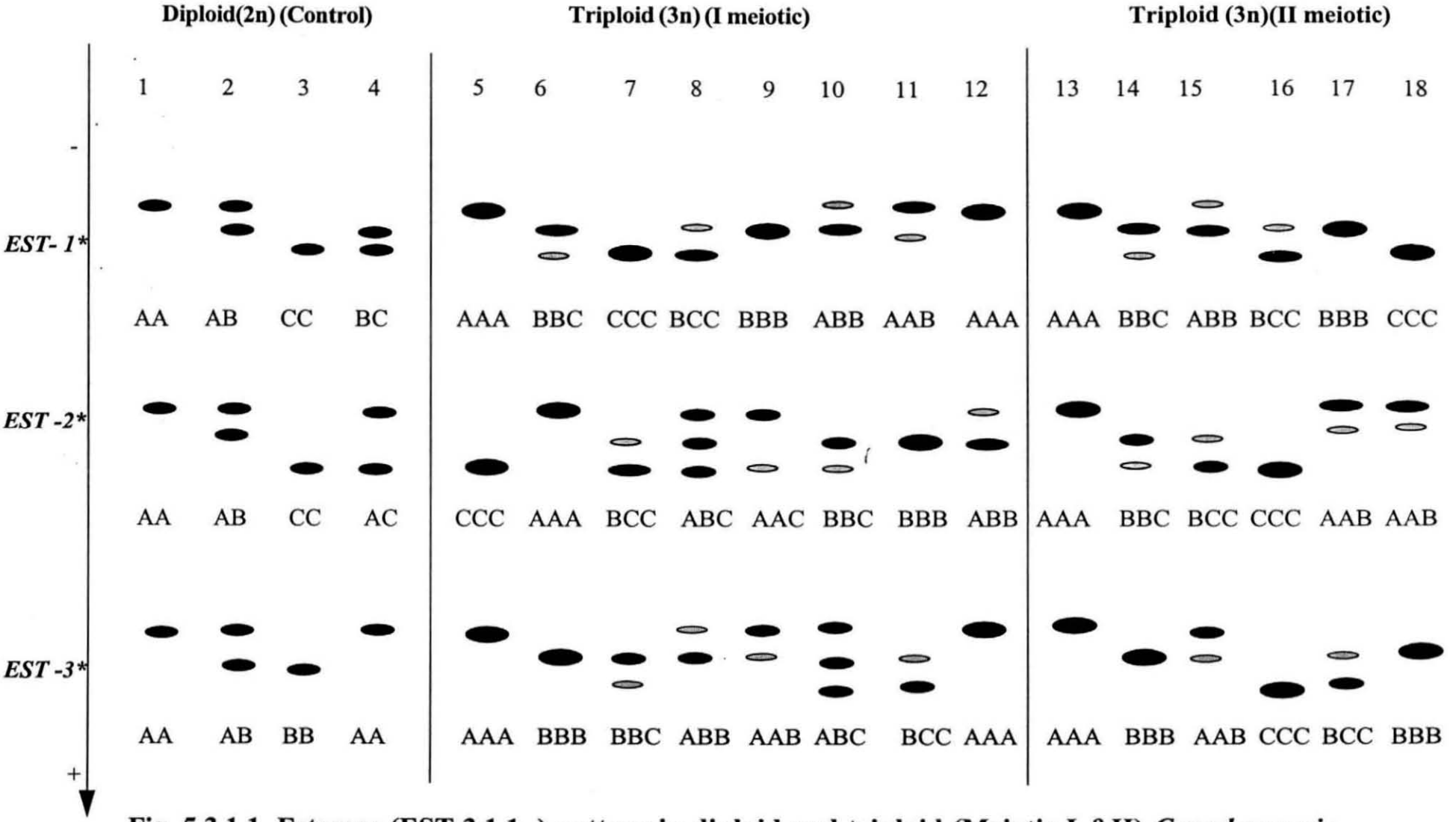


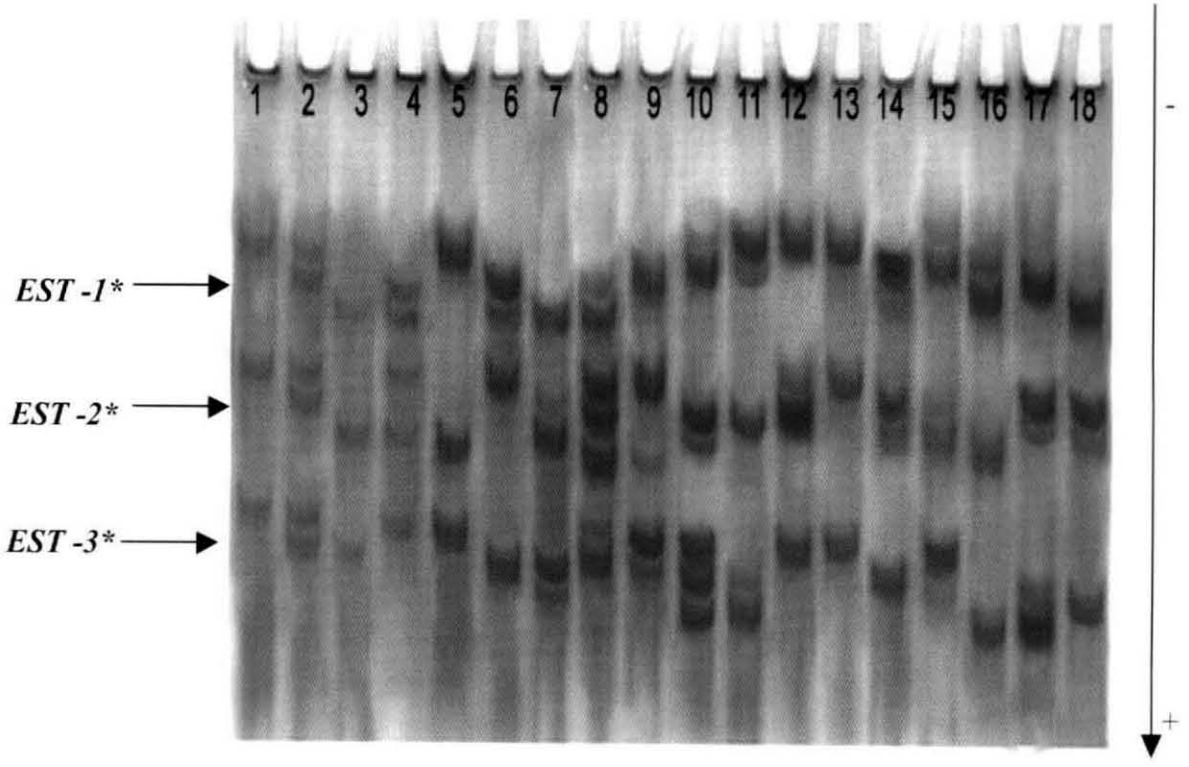
Fig. 5.3.1.1. Esterase (EST 3.1.1.-) pattern in diploid and triploid (Meiotic I &II) *C.madrasensis*

Plate 5.3.1

Zymogram patterns of Esterase in *C.madrasensis*

	2n (Control)	3n (I meiotic)	3n (II meiotic)
*EST-1**	AA =100/100 AB =100/120 CC =133/133 BC =120/133	AAA = 100/100/100 BBC = 120/120/133 CCC = 133/133/133 BCC = 120/133/133 BBB = 120/120/120 ABB = 100/120/120 AAB = 100/100/120 AAA = 100/100/100	AAA = 100/100/100 BBC = 120/120/133 ABB = 100/120/120 BCC = 120/133/133 BBB = 120/120/120 CCC = 133/133/133
*EST-2**	AA =100/100 AB =100/116 CC = 125/125 AC = 100/125	CCC = 125/125/125 AAA = 100/100/100 BCC = 116/125/125 ABC = 100/116/125 AAC = 100/100/125 BBC = 116/116/125 BBB = 116/116/116 ABB = 100/116/116	AAA= 100/100/100 BBC = 116/116/125 BCC = 116/125/125 CCC = 125/125/125 AAB = 100/100/116 AAB = 100/100/116
*EST-3**	AA =.100/100 AB =.100/109 BB = 109/109 AA =.100/100	AAA = 100/100/100 BBB = 109/109/109 BBC = 109/109/125 ABB = 100/109/109 AAB = 100/100/109 ABC = 100/109/125 BCC = 109/125/125 AAA = 100/100/100	AAA = 100/100/100 BBB = 109/109/109 AAB = 100/100/109 CCC = 125/125/125 BCC = 109/125/125 BBB = 109/109/109

Plate 5.3.1



Esterase (EST 3.1.1.-)

Lanes: 1-4 =Diploid(2n) Control; 5-12=Triploid(3n) I meiotic; 13-18 =Triploid(3n)II meiotic

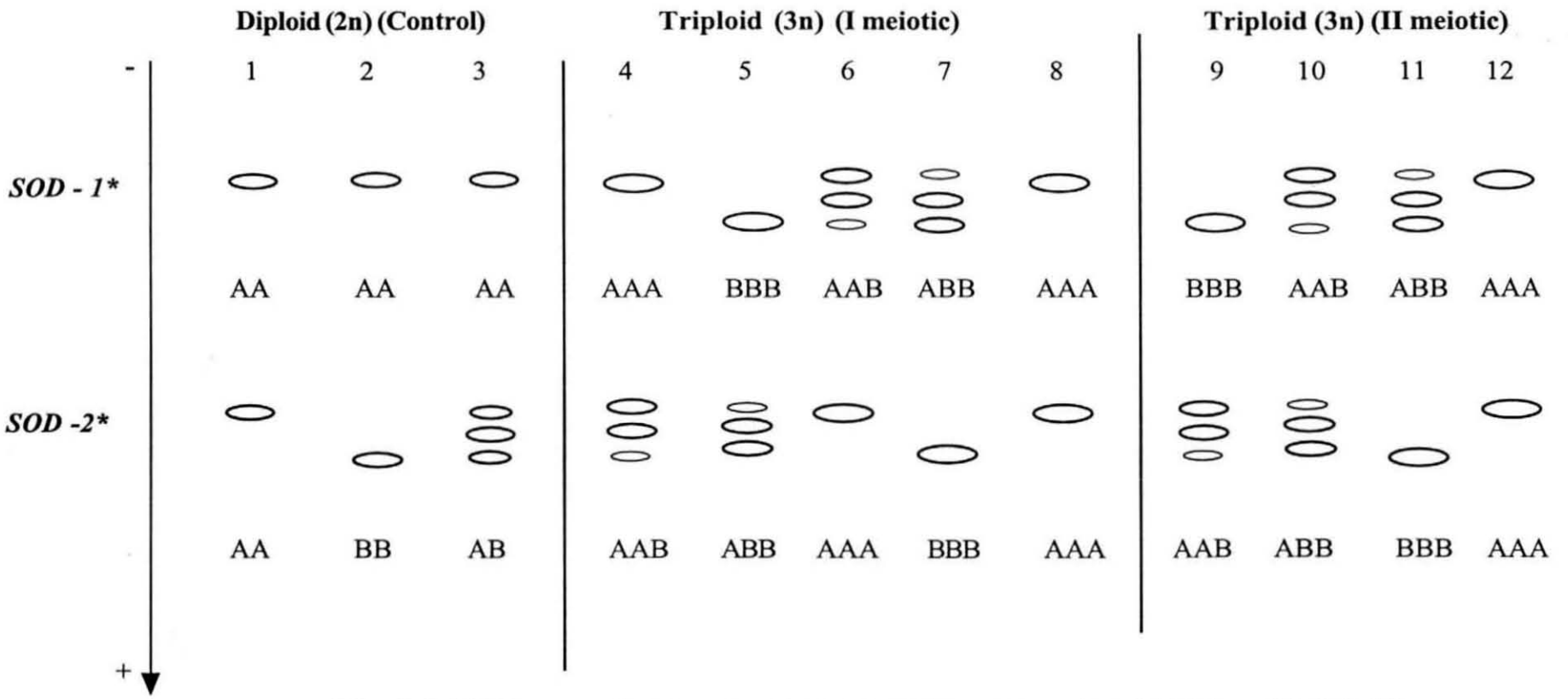


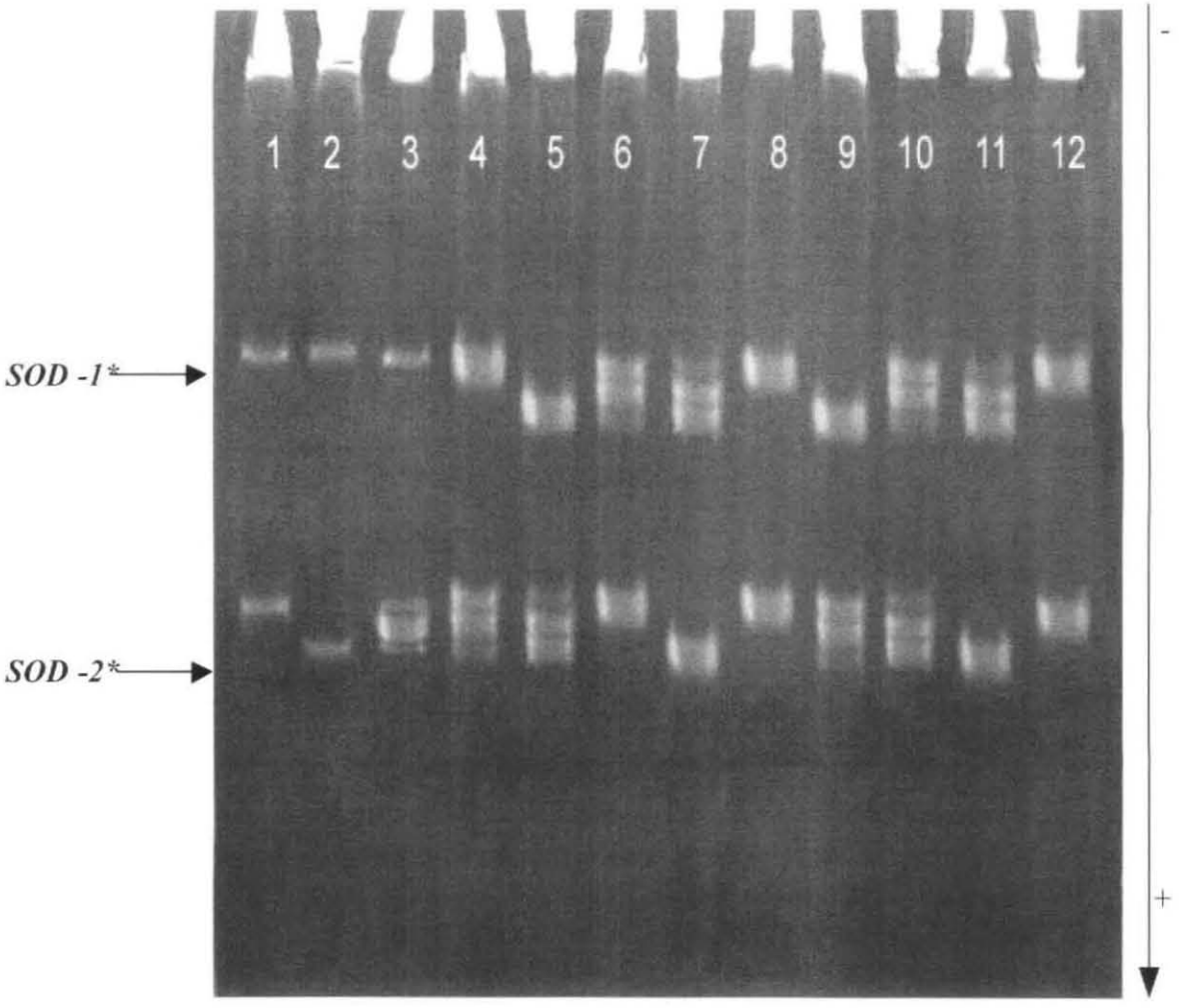
Fig. 5.3.1.2.Superoxide dismutase(SOD 1.15.1.1) pattern in diploid and triploid (Meiotic I &II) *C.madrasensis*

Plate 5.3.2

Zymogram patterns of Superoxide dismutase in *C.madrasensis*

	2n (Control)	3n (Imeiotic)	3n (II meiotic)
*SOD-1**	AA =100/100 (All 3 samples)	AAA= 100/100/100 BBB= 113/113/113 AAB= 100/100/100/100/113/113 ABB= 100/100/113/113/113/113 AAA= 100/100/100	BBB= 113/113/113 AAB=100/100/100/100/113/113 ABB= 100/100/113/113/113/113 AAA= 100/100/100
*SOD –2**	AA =100/100 BB =118/118 AB =100/118	AAB= 100/100/100/100/118/118 ABB= 100/100/118/118/118/118 AAA= 100/100/100 BBB= 118/118/118 AAA= 100/100/100	AAB= 100/100/100/100/118/118 ABB= 100/100/118/118/118/118 BBB= 118/118/118 AAA= 100/100/100

Plate - 5.3.2



Superoxide dismutase (SOD 1.15.1.1)

Lanes: 1-3 =Diploid(2n) Control; 4-8=Triploid(3n) I meiotic; 9-12=Triploid(3n)II meiotic

Glucose phosphate isomerase (GPI)

Herein two zones of activity were observed (Fig. 5.3.1.3). Since TG buffer gave the best results, other buffers were not tried. The *GPI-1** locus had two allele viz. 100 and 120 in triploids, only a single allele was present in the control. At *GPI-2**, two alleles were scored, a fast moving 100 and a slow moving 90. Like *SOD-1**, *GPI-1** was monomorphic in control but exhibited polymorphisms in both triploids (Plate 5.3.3a).

Phosphoglucomutase (PGM)

Only one zone of enzyme activity was present for PGM, which was apparently determined by a single gene locus (*PGM**) (Plate 5.3.3b). The different phenotypes at this locus indicated 3 alleles (100,133 and 166) and the band pattern observed for diploids and triploids are shown in Fig. 5.3.1.4.

The double-banded pattern of heterozygotes of both enzymes (PGM and EST) suggests that the structure of each enzyme is a monomer.

Glucose - 6 - phosphate dehydrogenase(G_6PDH)

G_6PDH gave two zones of enzymatic activity (Fig. 5.3.1.5), which were apparently determined by at least 2 gene loci (*G_6PDH-1** and *G_6PDH-2**) (Plate 5.3.4). The slow migrating enzymes located in zone I exhibited variability indicating polymorphism for this *G_6PDH-1** locus. The different phenotypes at this locus indicated 3 genotypes for control (i.e. 100/100; 100/144 and 144/144) and four genotypes for triploid i.e. 100/100/100; 100/100/100/100/144/144; 144/144/144 and 100/100/144/144/144/144 (Fig.5.3.1.6). The more anodal migrating zone II also exhibited variability producing similar phenotypes as zone I, i.e. 3 alleles for control and 4 alleles for triploids.

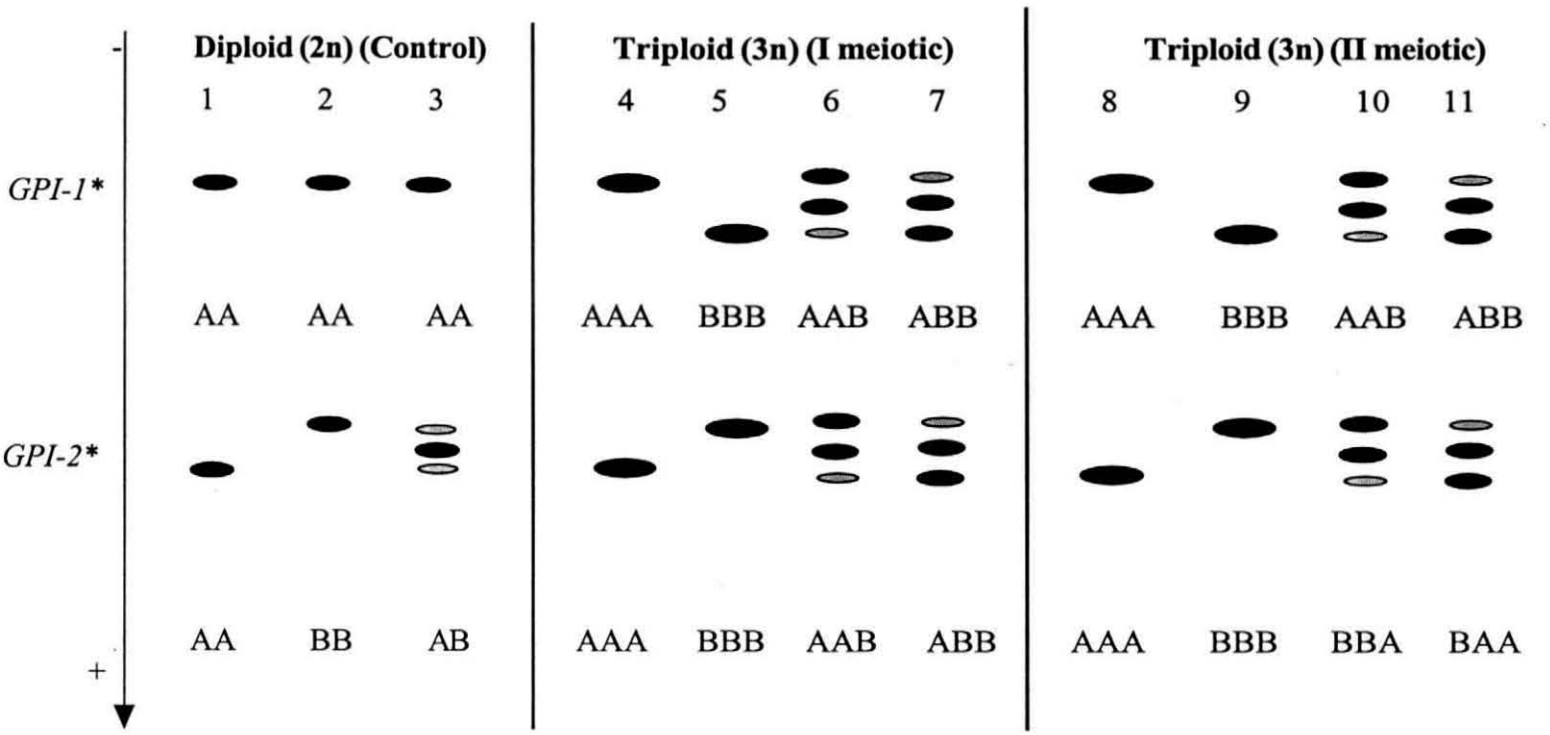


Fig. 5.3.1.3.Glucose phosphate isomerase(GPI 5.3.1.9.) pattern in diploid and triploid (Meiotic I &II) *C.madrasensis*

Plate 5.3.3

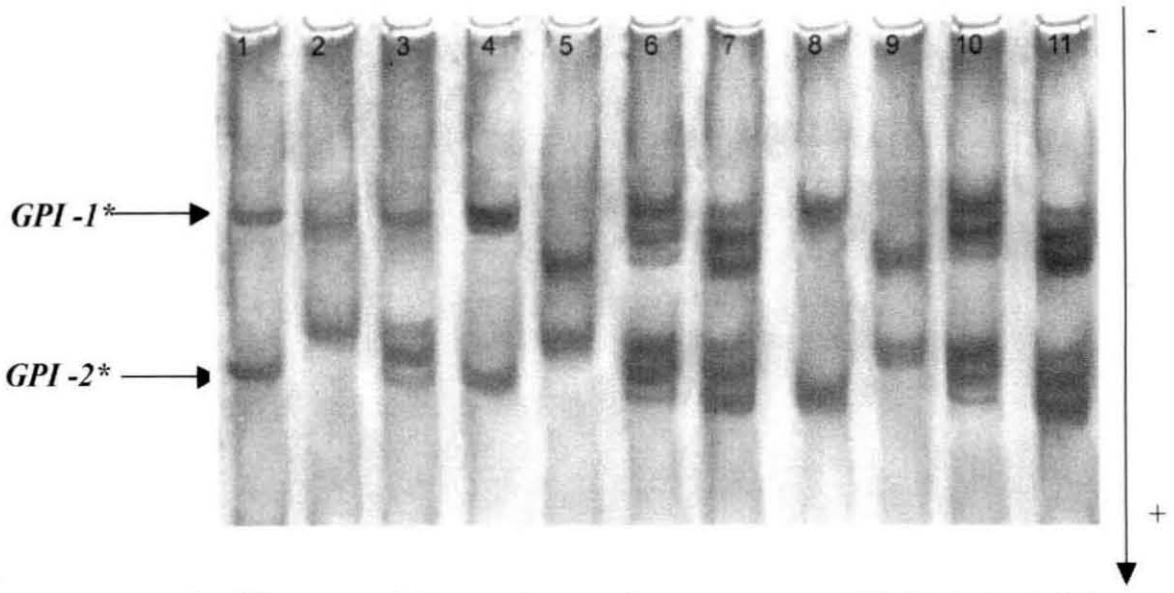
a) Zymogram patterns of Glucose phosphate isomerase in *C.madrasensis*

	2n (Control)	3n (Imeiotic)	3n (II meiotic)
*GPI-1**	AA = 100/100 (All 3 samples)	AAA= 100/100/100 BBB= 120/120/120 AAB= 100/100/100/100/120/120 ABB= 100/100/120/120/120/120	AAA= 100/100/100 BBB= 120/120/120 AAB= 100/100/100/100/120/120 ABB= 100/100/120/120/120/120
*GPI-2**	AA =100/100 BB =090/090 AB =100/90	AAA= 100/100/100 BBB = 090/090/090 BBA = 090/090/090/090/100/100 BAA= 090/090/100/100/100/100	AAA= 100/100/100 BBB = 090/090/090 BBA = 090/090/090/090/100/100 BAA = 090/090/100/100/100/100

b) Zymogram patterns of Phosphoglucomutase in *C.madrasensis*

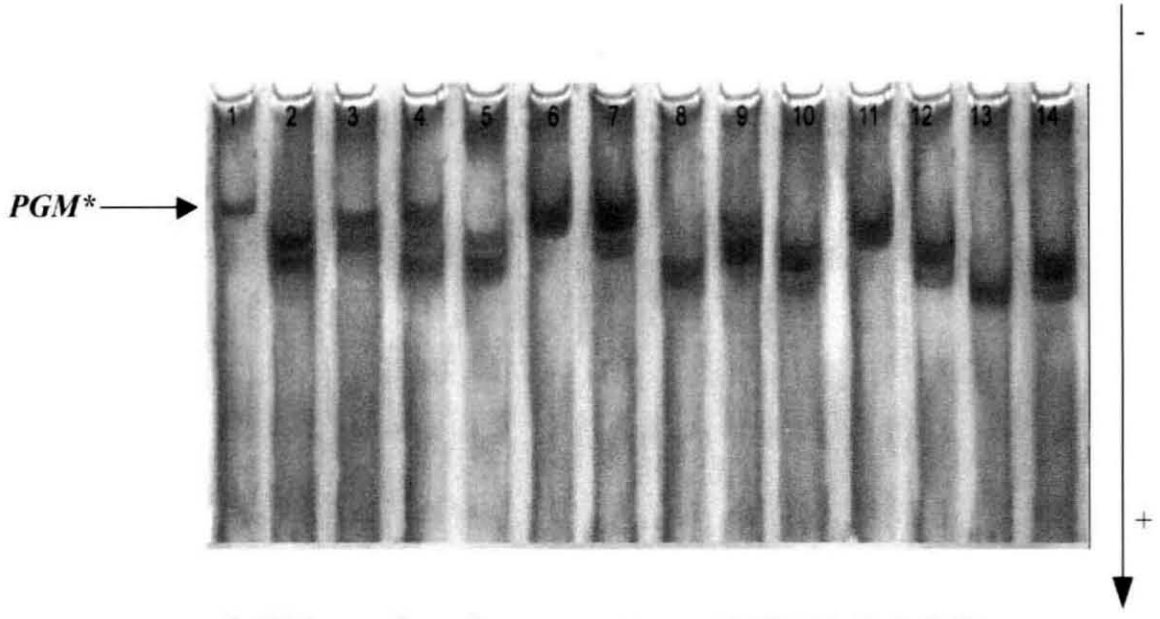
	2n (Control)	3n (Imeiotic)	3n (II meiotic)
*PGM**	AA =100/100 BC =133/166 AB =100/133 AC =100/166	BCC = 133/166/166 AAA= 100/100/100 AAB = 100/100/133 CCC = 166/166/166 ABB = 100/133/133 BBC = 133/133/166	AAA= 100/100/100 BBC = 133/133/166 CCC = 166/166/166 BBC = 133/133/166

Plate - 5.3.3



a) Glucose Phosphate Isomerase (GPI 5.3.1.9.)

Lanes: 1-3 =Diploid(2n) Control; 4-7=Triploid(3n) I meiotic; 8-11=Triploid(3n)II meiotic



b)Phosphoglucomutase (PGM 5.4.2.2)

Lanes: 1-4=Diploid(2n) Control; 5-10=Triploid(3n) I meiotic; 11-14=Triploid(3n)II meiotic

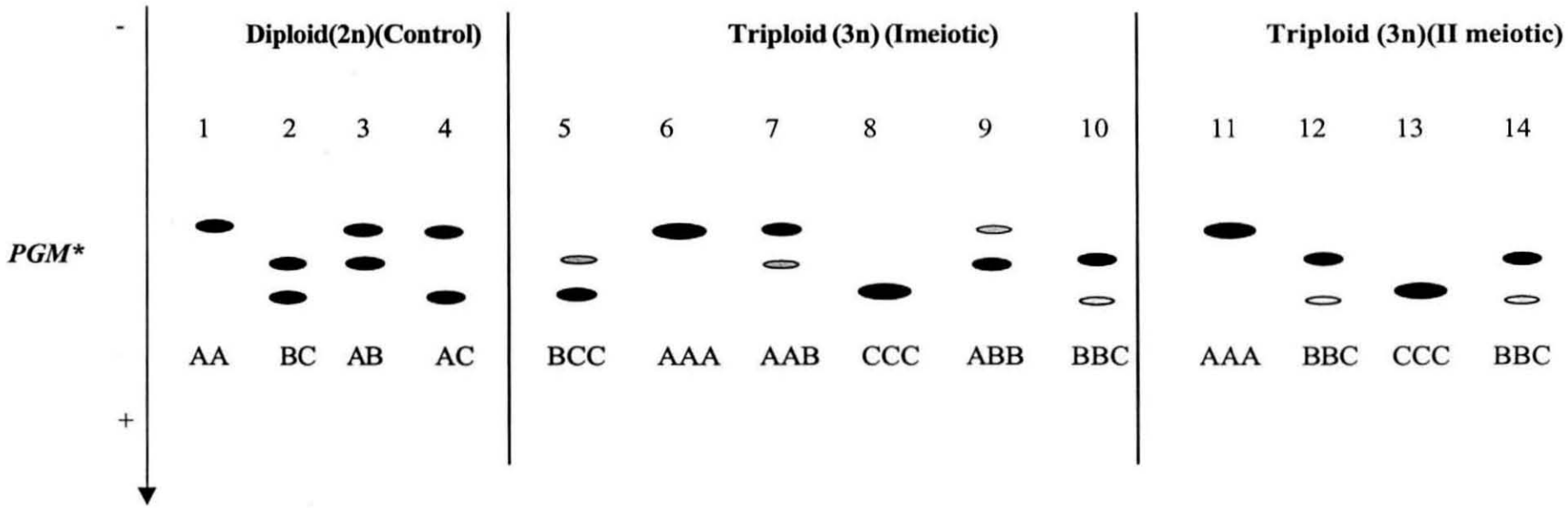


Fig. 5.3.1.4. Phosphoglucomutase (PGM 5.4.2.2) pattern in diploid and triploid (Meiotic I &II) *C.madrasensis*

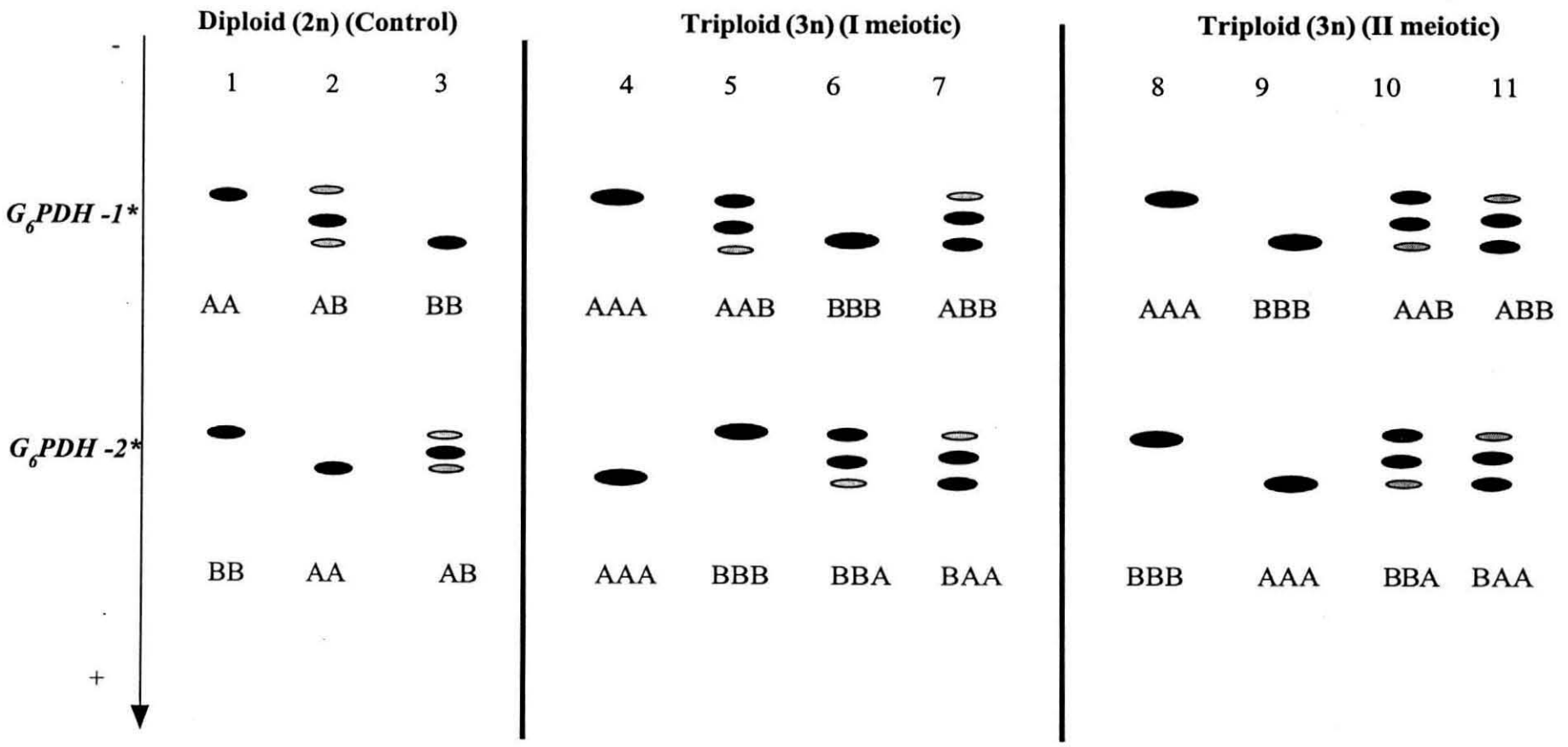


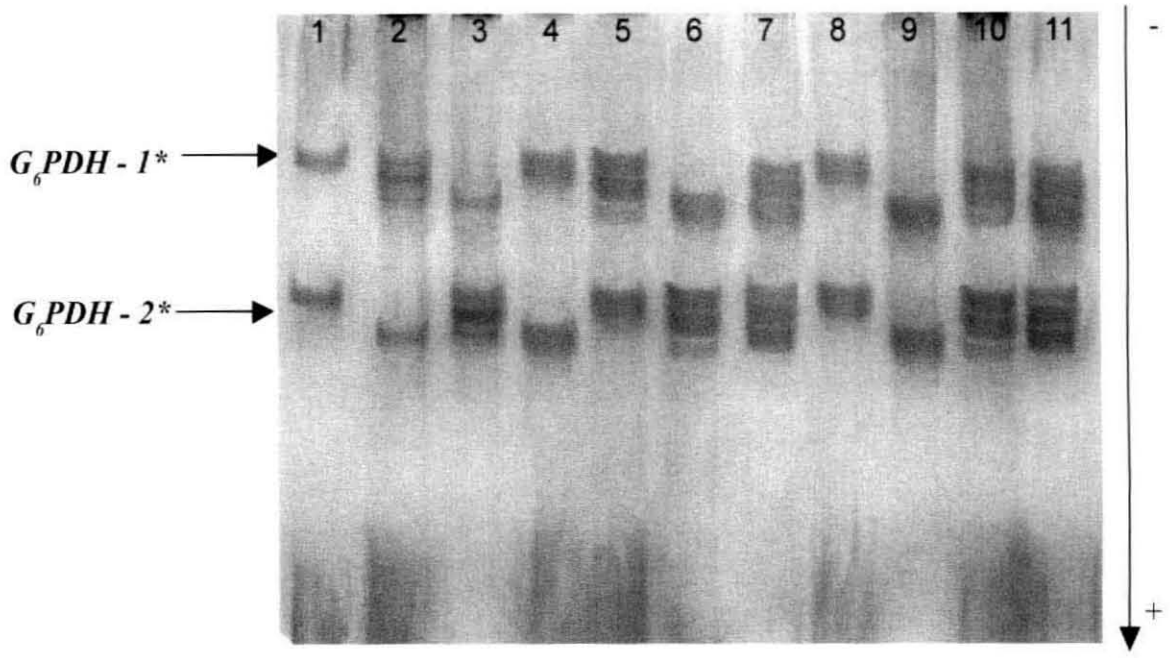
Fig. 5.3.1.5.Glucose-6-phosphate dehydrogenase (G_6PDH 1.1.1.49) pattern in diploid and triploid (MeioticI &II) *C.madrasensis*

Plate 5.3.4

Zymogram patterns of Glucose-6-phosphate dehydrogenase in *C.madrasensis*

	2n (Control)	3n (Imeiotic)	3n (II meiotic)
*G_6PDH- 1**	AA =100/100 AB =100/144 BB =144/144	AAA= 100/100/100 AAB= 100/100/100/100/144/144 BBB= 144/144/144 ABB= 100/100/144/144/144/144	AAA= 100/100/100 BBB= 144/144/144 AAB= 100/100/100/100/144/144 ABB= 100/100/144/144/144/144
*G_6PDH- 2**	BB =075/075 AA =100/100 AB =075/100	AAA= 100/100/100 BBB= 075/075/075 BBA= 075/075/075/075/100/100 BAA= 075/075/100/100/100/100	BBB= 075/075/075 AAA= 100/100/100 BBA= 075/075/075/075/100/100 BAA= 075/075/100/100/100/100

Plate - 5.3.4



Glucose-6-Phosphate dehydrogenase(G_6PDH 1.1.1.49)

Lanes: 1-3 =Diploid(2n) Control; 4-7=Triploid(3n) I meiotic; 8-11=Triploid(3n)II meiotic

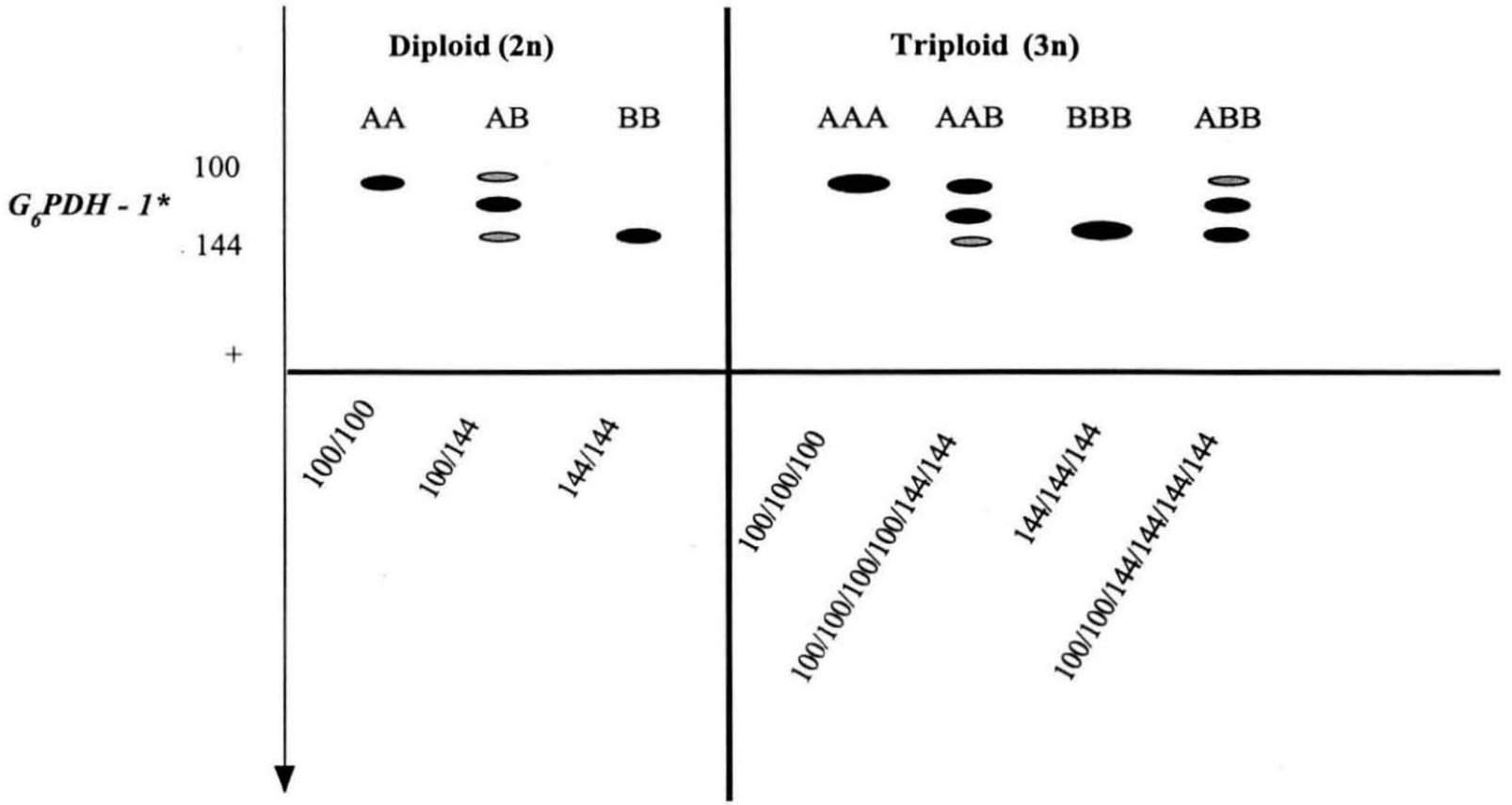


Fig. 5.3.1.6. Zymogram pattern of Genotype and representation of dimeric enzymes in diploid and triploid (*eg. G_6PDH -1) ; diploid heterozygotes exhibit staining intensities in ratios 1:2:1 and triploids 4:4:1/1:4:4**

Examples of observed diploid and triploid phenotypes of all 5 enzymes (10 polymorphic loci) are shown in Fig. 5.2.4.1. Triploids exhibited a higher degree of heterozygous phenotypes for enzymes having subunit structures derived from a single locus. For monomeric loci such as *EST-1*, 2* & 3** and *PGM**, the two bands stained with equal intensity in diploids. In triploids the allelic product under the control of the duplicated portion of the genome exhibited a proportionate increase in staining intensity, producing a two-banded pattern displaying a 2:1 ratio. Dimeric enzymes (e.g. G_6PDH) produced triploid phenotypes staining in a 4:4:1 ratio (Fig. 5.3.1.6).

5.3.2. Allelic frequency

Allelic frequencies were estimated at the 10 loci belonging to 5 different enzymes. (EST, GPI, G_6PDH, PGM and SOD) as in Table 5.3.2.1. Comparison of allele frequencies reveals that all alleles of three groups were polymorphic except at 2 loci (*SOD1* and GPI 1**) of control. The frequencies at 10 polymorphic loci shows that the values differed much between each group.

5.3.3.Heterozygosity

The average heterozygosities, both observed and expected were estimated for each group. The heterozygosity of 10 polymorphic loci in three groups are presented in Table 5.3.3.1. Heterozygosity varied greatly among loci and among groups. When all loci were combined, the triploids showed much higher levels of heterozygosity than did normal diploids. The average expected heterozygosity values for I meiotic triploids (MI), II meiotic triploids (MII) and control (C) were 0.54, 0.56 and 0.31 respectively, while the average observed heterozygosity for the above groups were 0.59,0.57 and 0.18 respectively.

Table 5.3.2.1. **Allele frequencies of isozymes in adductor muscle of *C. madrasensis***

Locus	Allele	I Meiotic triploid	II Meiotic triploid	Diploid
*EST-1**	100	0.41	0.43	0.73
	120	0.41	0.40	0.10
	133	0.18	0.17	0.17
*EST-2**	100	0.28	0.47	0.58
	116	0.43	0.28	0.25
	125	0.27	0.25	0.17
*EST-3**	100	0.44	0.42	0.63
	109	0.37	0.40	0.37
	125	0.16	0.16	0
*G_6PDH-1**	100	0.60	0.63	0.85
	144	0.40	0.37	0.15
*G_6PDH-2**	100	0.41	0.36	0.63
	75	0.59	0.64	0.36
*GPI-1**	100	0.52	0.59	1
	120	0.48	0.41	0
*GPI-2**	100	0.62	0.62	0.60
	90	0.38	0.41	0.40
*PGM**	100	0.44	0.37	0.87
	133	0.35	0.31	0.07
	166	0.21	0.32	0.07
*SOD-1**	100	0.56	0.51	1
	113	0.44	0.49	0
*SOD-2**	100	0.67	0.39	0.87
	118	0.33	0.44	0.13

Table 5.3.3.1. **Heterozygosity of allozymes in *C.madrasensis***

Loci	I Meiotic		II Meiotic		Diploid	
	Ho	**He**	**Ho**	**He**	**Ho**	**He**
*EST-1**	0.66	0.63	0.63	0.62	0.23	0.42
*EST-2**	0.70	0.66	0.60	0.64	0.56	0.57
*EST-3**	0.66	0.64	0.64	0.63	0.26	0.46
*G_6PDH-1**	0.70	0.48	0.50	0.46	0.10	0.25
*G_6PDH-2**	0.53	0.48	0.60	0.46	0.13	0.46
*GPI-1**	0.36	0.49	0.47	0.45	0	0
*GPI-2**	0.56	0.47	0.56	0.44	0.20	0.48
*PGM**	0.70	0.64	0.60	0.66	0.23	0.24
*SOD-1**	0.53	0.49	0.60	0.49	0	0
*SOD-2**	0.50	0.44	0.43	0.65	0.06	0.23
Average	0.59	0.54	0.57	0.55	0.18	0.31

The allelic heterozygotes (i.e. A, B & C) were observed at the *EST-2** and *EST-3** loci in MI and *EST-3** locus of MII. All other heterozygotes present in triploids were diallelic (e.g. AAB, ABB).

5.3.4.Statistical Analysis

Both single and multiple–locus heterozygosities for enzyme loci in each ploidy class were summarized with the results of statistical comparisons in Table 5.3.4.1. Paired comparison "t" tests indicated that single locus heterozygosity was consistently higher in both meiosis I & II triploids than in diploids (Table 5.3.4.2). But there was no associated difference between Meiosis I & II triploid siblings.

A simple "t" test established that multiple locus heterozygosity was significantly greater among both meiotic I &II triploids than among diploids ($P<0.0005$). However there was no significant difference between meiosis I & II triploids ($P > 0.1$) (Table 5.3.4.2).

5.4.Discussion

The present study demonstrates that allozyme electrophoresis is an efficient method with high resolution for estimating the efficacy of experimental induction of triploidy in *C.madrasensis*. All the allozymes used in the present study were useful for diagnosis of ploidy in the species because it allowed direct visualization of gene duplication at discrete structural gene loci. Allen *et al.* (1982) found that differences in staining intensity were sufficient to distinguish diploid and triploid phenotypes. The present work supports their observation that induction of polyploidy leads to a substantial increase in heterozygosity in the resultant generation of polyploid individuals (Table 5.3.3.1).

Increased heterozygosity per triploid individual indicates its usefulness as additional tool for confirmation of ploidy level.

Table 5.3.4.1. **Single - locus heterozygosities (pooled) at ten polymorphic allozyme loci, including the average (±SE)multiple-locus heterozygosity for all six loci, in different ploidy classes of *C. madrasensis***

Ploidy Class	**Single - locus heterozygosity (Ho)**										**Multiple-locus heterozygosity**
	*Est1**	*Est2**	*Est3**	*PGM**	*G_6PDH1**	*G_6PDH2**	*GPI1**	*GPI2**	*SOD1**	*SOD2**	
Normal diploids (N=30)	0.23	0.57	0.27	0.23	0.10	0.13	0	0.20	0	0.07	0.080±0.01
Meiotic I triploids (N=30)	0.67	0.70	0.67	0.70	0.70	0.53	0.53	0.50	0.37	0.57	0.571±0.06 t value =7.63 df = 58 P<0.0005
Meiotic II triploids (N=30)	0.63	0.60	0.63	0.60	0.57	0.60	0.47	0.57	0.60	0.43	0.58±0.060 t value =8.08 df = 58 P<0.0005

Table 5.3.4.2. **Paired "t" test indicating levels of heterozygosity.**

	t-value	**df**	**Significant level**
Single locus heterozygosity			
I Meiotic & Diploid	10.632	9	P<0.0001
II Meiotic & Diploid	8.460	9	P<0.0001
I Meiotic & II Meiotic	0.729	9	P>0.05
Multiple locus heterozygosity			
I Meiotic & Diploid	7.627	58	P<0.0001
II Meiotic & Diploid	8.077	58	P<0.0001
I Meiotic & II Meiotic	0.054	58	P>0.1

Confirmation of ploidy facilitated comparisons of performance between distinct ploidy classes similar to that of Hawkins *et al.* (2000). Most of the enzyme loci studied had few alleles, and a triploid animal most of the time appeared to have 2 bands with one of the bands having stronger staining intensity in the heterozygote condition. Allozyme scorings yielded reliable results in which it was possible to distinguish between the two possible heterozygotes in a triploid animal (e.g. between AAB and ABB).

Heterozygosity was much higher in triploids than in diploids for all the 10 loci examined. Differences in heterozygosity between diploids and both types of triploids were statistically significant. But there was no difference between heterozygosity of I and II meiotic triploids in the present study. Beaumont (2000) explained the effect of recombination at a polymorphic locus to clarify the genetic consequences of triploidy induced at Meiosis I or Meiosis II. According to him, complete recombination at a heterozygous locus in the female progenitor, would give either homozygous or heterozygous diploid eggs allowing MI suppression, but exclusively heterozygous diploid eggs if Meiotic II was suppressed. Therefore, at loci with high recombination frequencies, MII triploids were likely to be more heterozygous than MI triploids. Beaumont *et al.* (1995) indicated that many of the commonly screened allozyme loci had high recombination frequencies, which could be the reason for the high heterozygosity expressed with almost similar magnitude in both types of triploids in the present study.

One of the purposes of the isozyme evaluation was to examine the hypothesis that MI triploids may have greater heterozygosity leading to enhanced growth compared to MII triploids. Whereas significantly higher growth of Meiotic I triploids than the Meiotic II triploids or diploids has been reported for the two year old *C.virginica* (Stanley *et al.*, 1984), enhanced heterozygosity was reported for the 15 month old *Ostrea edulis*

(Hawkins *et al.,* 1994) and velliger larvae of *C.gigas* and *Mytilus edulis* (Yamamoto *et al.,* 1988; Beaumont and Kelly, 1989). Other studies failed to demonstrate any significant differences between the two types of triploids as in *C.gigas* and *Mya arenaria* (Downing and Allen, 1987; Mason *et al.,* 1988; Shpigel *et al.,* 1992). Hawkins *et al.* (2000) established that multilocus enzyme heterozygosity was significantly greater among Meiosis I triploids than among diploids ($P<0.0001$) with no difference between I & II triploids ($P>0.05$). This observation confirms to the present observation that both meiotic I and II triploids expressed higher multilocus heterozygosity than did diploids ($P<0.0005$) with no significant differences between meiosis I and II triploids.

Meiotic I triploids which were significantly bigger than meiotic II triploid groups in *C.madrasensis* did not show increased heterozygosity at all the 10 loci analysed. Interestingly, there was no detectable or real influence of heterozygosity *per se* on body size within both types of triploids in the present study. Further studies are required to elucidate the genes responsible for body growth in *C.madrasensis* other than heterozygosity.

CHAPTER 6

GROWTH

6.1.Introduction

Since the initiation of triploidy induction in oysters by Stanley *et al.* (1981), many studies have been carried out in production of triploids indicating their advantages in growth and marketing over diploid siblings (Tabarini, 1984; Allen and Downing, 1986; Mason *et al.*, 1988 and Komaru and Wada, 1989). Beaumont and Kelly (1989) and Utting *et al.* (1996) reported significantly higher shell length of triploid larvae. Matthiessen and Davis (1992) reported that triploid American oyster *Crassostrea virginica* survived better than diploid oysters exposed to the parasite *Haplosporidium nelsoni.* In assessing triploidy in oysters, Gardner *et al.* (1996) indicated greater adductor muscle index in triploid oysters than in diploids. Substantial size differences of the adductor muscle between diploid and triploid Pacific oysters (*C.gigas*) have also been reported (Akashige, 1990). Nell *et al.* (1994), comparing the performances of triploid *Saccostrea commercialis* over diploid siblings, observed higher dry meat weight, higher condition index and quicker growth rate in triploids. Reduction of grow out time was another advantage of triploids (Graham, 1991) producing a 20% improvement in labour cost. Better survival of triploid hatchery stocks with better meat condition during winter compared to wild diploids can further improve the profitability in oyster farms (Hand *et al.*, 1998).

Studies on these aspects on triploidy in the Indian backwater oyster *Crassostrea madrasensis* have not been made so far. In this study, morphometric characters including length, breadth, weight, condition index, gonadal development, dry meat weight and adductor muscle diameter were evaluated for triploids of *C.madrasensis.* The performance of growth of triploid larvae and spat were compared with the diploid controls.

6.2.Materials and methods

Fertilized eggs were incubated with 6-DMAP for 8-minutes duration for inducing I meiotic (M I) and II meiotic (M II) triploids. Untreated fertilized diploid eggs were reared as control. For each treatment, triplicates were maintained and the larvae were reared in 40 l plastic trough at a larval density of 5 larvae/ml. Filtered seawater (100%) was changed on alternate days while 50% seawater was exchanged daily. The feed (*Isochrysis galbana)* was cultured following a serial dilution technique (Gopinathan, 1982). Feeding rate of the larvae at various stages was adopted from Nayar *et al.* (1987). The water temperature ranged from 29 to 31°C and salinity from 31 to 33 ppt during the period of study. The pH was 8.1 to 8.2. Gentle aeration was given to the larvae (Plate.6.2.1).

On 3rd, 6th, 9th, 12th and 15th day of post fertilization, 20 larvae were fixed in 1% formalin and anterior posterior axis measurement (length) was taken through precalibrated micrometer at 150X magnification.

When the eyed stage was attained, the inner side and bottom of the rearing trough was fixed with polythene sheet for spat settlement. After settlement, the spat were reared for three weeks in troughs by providing mixed algae consisting of *Chaetoceros* sp., *Thalassiosira* sp. and *Nitzchia* sp. After rearing for 3 weeks, the spat removed from the polythene sheet were kept in separate box-type rearing cages of 40x40x10 cm webbed with synthetic twine and encircled with velon screen. Cages were suspended from a rack erected in the intertidal area (Plate 6.2.2).

Young oysters were reared for a year. Once every 3 months, 20 oysters were measured for length and breadth using Vernier calipers. Weight was taken to the nearest of 0.01g in a Sartorius balance. For a sample of 10 oysters in each treatment, the diameter of the adductor muscle was noted. Condition Index denotes the degree of fatness of an oyster or the extent to which the meat fills the shell cavity. Condition Index (C.I) was estimated

Plate 6.2.1

Plate 6.2.2

following the method adopted by Crosby and Gale (1990) by using the equation

$$C.I - \frac{\text{Dry meat weight (g)}}{\text{Cavity volume (g)}} \times 1000$$

Where, Cavity volume = whole weight(g) – shell weight (g) (Lawrence and Scott, 1982). The dry weight of the meat was recorded after keeping the meat in a hot air oven at 60°C for 48 hrs. Gonadal development was evaluated macroscopically during the period of the experiment.

The mean values of the observations made for each treatment were statistically analyzed using SYSTAT 7.0.

6.3.Results

Larval growth

Diploid larvae attained 51.5 ± 3.9µm on the third day whereas the M II reached 58.1± 4.5µm and M I attained 79.7± 2.21µm. On the ninth day, 162 ± 6.9µm was attained by M I larvae whereas the M II and diploid larvae had mean length of 132.8 ± 4.3 and 131.1± 5.8µm respectively. On day 12, the M I larvae had greater mean length of 205.8 ± 5.1µm than MII (159.4 ± 3.7µm) and diploid (152.7± 4.8µm) larvae. On day 15, the length of the diploid larvae was only 194.2 ± 5.3µm whereas M II and M I larvae attained 237.4 ± 11.08 and 262.3 ± 10.5µm, respectively (Table 6.3.1). The daily larval growth rate for diploid, M II and M I was 12.9, 15.8 and 17.5µm, respectively.

Larval survival

Survival of the diploid larvae on the first day was 92.06 ± 0.8% which came down to 6.69 ± 0.45% on day 20. Final survival of 5.25 ± 1.06% was observed for M II triploids. M I larvae had less survival of 4.55 ± 0.68% (Table 6.3.2).

Juvenile Growth

Length: Progressive increase in the length of the oysters is given in Table 6.3.3. From a mean length of 7.1 mm, the diploid oysters had grown to 18.7 ± 1.76, 34.6 ± 2.83 and 40.1 ± 3.38 mm

Table 6.3.1. **Comparison of length of triploids and diploid larvae of *C. madrasensis***

Length*(µm)			
Day	Treatment		
	I meiotic	II meiotic	Diploid
3	79.7 ± 2.21	58.1 ± 4.48	51.5 ± 3.88
6	83 ± 4.95	64.7 ± 2.98	71.4 ± 3.54
9	162.7 ± 6.91	132.8 ± 4.29	131.1 ± 5.78
12	205.8 ± 5.07	159.4 ± 3.67	152.7 ± 4.82
15	262.3 ±10.45	237.4 ± 11.08	194.2 ± 5.34

*All values are expressed as mean ± SE

Table 6.3.2. **Survival percentage of triploids and diploid larvae of *C. madrasensis***

Survival (%)			
Group/day	I meiotic	II meiotic	Diploid
1st	86.90±1.82	85.71±1.38	92.06±0.79
2nd	50.70±0.68	48.62±0.78	56.46±0.33
4th	34.72±0.54	33.53±0.74	50.99±0.16
6th	17.71±0.22	17.46±0.18	32.89±0.32
20th (spat)	4.55±0.68	5.25±1.06	6.69±0.45

*All values are expressed as mean ± SE

in the 3rd, 6th and 9th months respectively. In the 12th month, the mean size attained was 43 ± 3.33 mm with a growth rate of 2.99 mm/month. The second meiotic oysters attained 24.2 ± 2.58, 35.5 ± 2.27 and 39 ± 1.82 mm in the 3rd, 6th, and 12th month, respectively and registered a growth rate of 2.45mm/month. The first meiotic triploids had grown to 27.1 ± 1.83, 37.9 ± 2.49 and 55.7 ± 5.01 mm in the 3rd, 6th, and 12th month, respectively with a growth rate of 3.87 mm/month (Plate 6.3.1).

Breadth: In terms of breadth, the diploid oysters had grown from 5.8±0.3 mm to 27.5 ± 2.09 and 36.3 ± 3.31 mm in the 6th and 12th month. The mean breadth of II meiotic triploid was 29.0 ± 2.36 and 34.8 ± 4.45 mm in the 6th and 12th month respectively. The I meiotic triploids attained a mean breadth of 34 ± 2.36 and 50.3 ± 3.85 mm in 6th and 12th month respectively (Table 6.3.3). The growth rate in breadth was 3.6 mm/month.

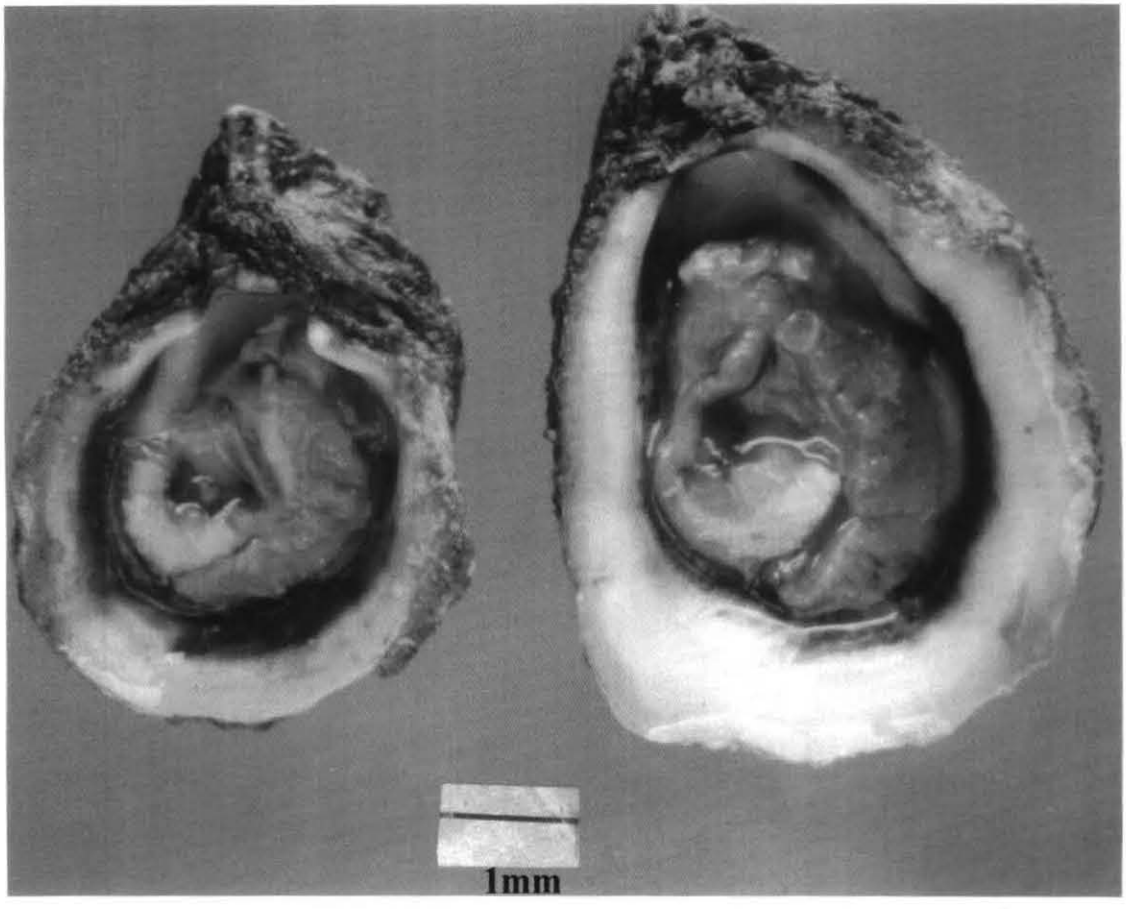
Weight: In the diploid, the mean weight was 9.4 ± 1.84 g in the 12th month. The weight increase was 0.78g/month. The mean weight of II meiotic oysters was 8.6 ± 2.76 g and the weight increase was 0.68 g/month. The I meiotic oyster had higher shell-on weight of 18.3 ± 2.87 g with rate of weight increase being 1.5 g/month (Table 6.3.3) (Plate 6.3.1).

Adductor muscle diameter (AMD): The diploid oyster had initial diameter of 2.9 mm, which increased to 4.2 and 4.3 mm in the 6th and 9th months respectively. At 12th month, the AMD ranged from 4.15 to 6.45 mm with an average of 5.6 mm.

In the II meiotic oysters, the initial AMD was 4mm. It increased to 5.8, 6.6 and 9.8mm in the 6th, 9th and 12th month respectively.

In the I meiotic oysters initial diameter increased from 4.12 mm to 6.5 mm in the 6th month, the average diameter was 8.3 mm. At the end of the experiment, i.e. at 12th month, the maximum AMD was 16.58mm and the minimum was 14.45mm with an average of 15.5 mm (Table 6.3.4: Fig. 6.3.1).

Plate 6.3.1



Diploid **Triploid**

12 months old diploid and triploid *C.madrasensis*

Table 6.3.3. **Comparison of growth in length, breadth and shell-on weight of diploid and triploid *C. madrasensis* (Mean ± SE)**

	Length (mm)			Breadth (mm)			Weight (g)		
Months After treatment	**I meiotic**	**II meiotic**	**Diploid**	**I meiotic**	**II meiotic**	**Diploid**	**I meiotic**	**II meiotic**	**Diploid**
2	9.3±0.61	9.6±0.57	7.1±0.41	7.1±0.49	7.6±0.51	5.8±0.30	0.1±0.028	0.4±0.02	0.05±0.005
3	27.1±1.83	24.2±2.58	18.7±1.76	23.8±1.82	21.6±2.9	16.6±1.52	2.5±0.33	2.3±0.53	1.1±0.21
4	35.1±2.21	31.8±2.24	31.3±2.51	29.7±2.31	27.4±2.65	24.4±2.13	5.3±0.70	4.9±0.90	3.3±0.64
5	37.4±2.39	34.5±2.38	33.2±2.78	33.2±2.47	29.5±2.70	25.6±2.09	6.0±0.80	5.6±1.03	4.1±0.77
6	37.9±2.49	35.5±2.27	34.6±2.83	34.0±2.36	29.0±2.36	27.5±2.09	6.5±0.88	5.6±1.04	4.2±0.80
7	39.1±2.67	39±1.54	38.8±3.68	32.6±2.77	28.1±2.23	32.1±2.84	7.0±1.05	5.5±1.02	6.0±1.26
8	41.7±3.17	32.5±2.23	40.2±3.56	33.5±2.91	26.6±2.32	31.1±2.53	8.0±1.40	4.5±0.89	6.7±1.31
9	41.9±3.32	32.8±2.27	40.1±3.38	32.5±2.82	25.9±2.03	31.3±2.71	8.1±1.41	4.6±0.88	6.6±1.40
10	45.0±3.51	29.9±2.47	40.8±4.0	35.5±2.81	25.1±2.67	32.1±2.74	10.6±1.85	4.1±0.81	7.6±1.57
11	51.7±4.05	38.3±2.28	46.2±4.30	45.5±4.01	33.1±3.71	38.5±4.47	15.5±2.72	6.9±2.01	10.4±2.58
12	55.7±5.01	39.0±1.82	43.0±3.33	50.3±3.85	34.8±4.45	36.3±3.31	18.3±2.87	8.6±2.76	9.4±1.84

Table 6.3.4. **Adductor muscle diameter, dry weight, Condition Index and Gonadal development in triploids and diploid *C.madrasensis***

	January02	April02	July02	October02
Adductor Muscle Diameter (mm)				
I Meiotic	4.1±0.38	6.5±0.54	8.3±0.56	15.5±1.07
II Meiotic	4.0±0.64	5.8±0.42	6.6±1.06	9.8±2.54
Diploid	2.9±0.49	4.2±0.49	4.3±0.37	5.6±0.92
Dry weight (g)				
I Meiotic	0.07±0.03	0.12±0.02	0.52±0.05	1.45±0.03
II Meiotic	0.03±0.02	0.10±0.05	0.23±0.03	0.86±0.07
Diploid	0.02±0.02	0.04±0.01	0.10±0.05	0.19±0.02
Condition Index				
I Meiotic	69.2	71.6	121.2	133.5
II Meiotic	63.8	66.9	55.3	108.5
Diploid	50.3	39.1	53.8	88.7
Gonadal development				
I Meiotic	Not distinct	Not distinct	Rudimentary gonads	Non functional shrunken gonads
II Meiotic				
Diploid	Not distinct	Immature	Mature ovary & testis	Ripe gonads

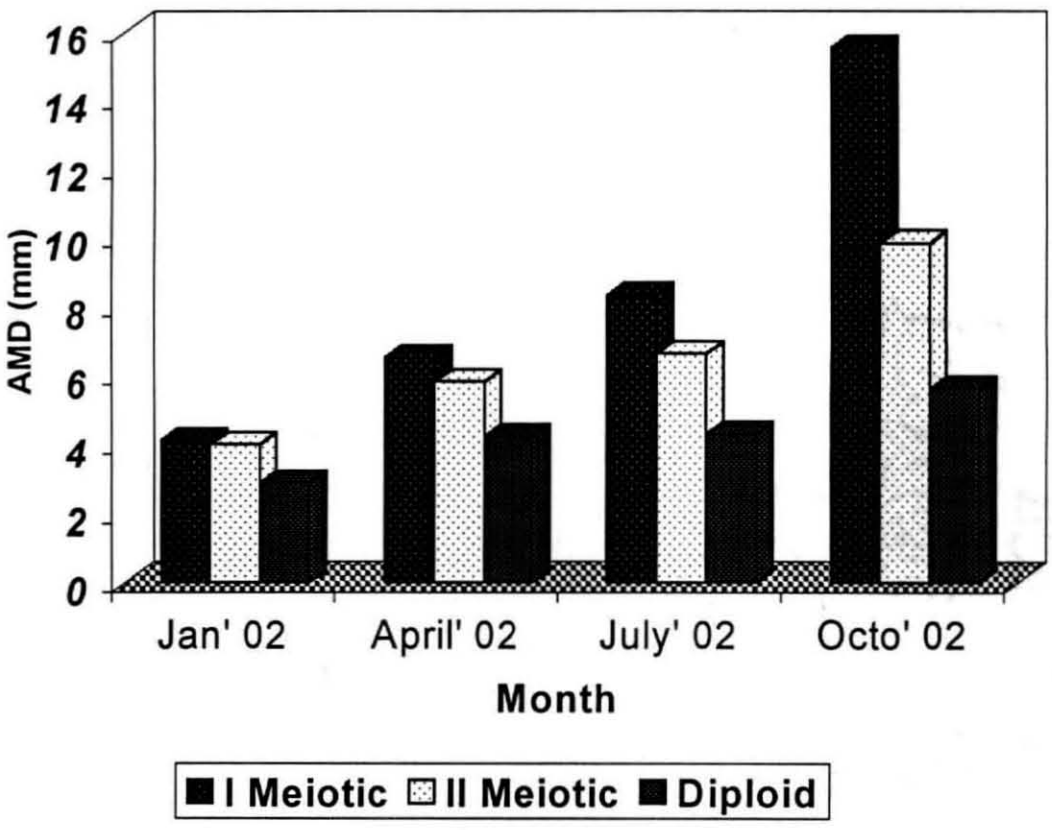


Fig. 6.3.1. Adductor Muscle diameter (AMD) of triploids and diploid *C. madrasensis*

Dry meat weight: Dry meat weight of diploid oysters increased from 0.02g to 0.19g in 12 months. The dry meat weight of II meiotic triploids ranged from 0.01 to 0.05 g with a mean of 0.03g. On month 12, it attained the maximum weight of 1.26g. The I meiotic triploids from initial average dry meat weight of 0.07g increased to 1.45 g with a maximum dry meat weight of 1.86g (Table 6.3.4).

Gonadal development: In January '02 both triploids and diploids were inactive sexually and afterwards diploids were identified as male or female by the appearance of developing ovaries and testis. In the month of July, the gonads of diploid oysters appeared densely packed. Female gonads were more easily discernible under the microscope in a squash preparation. Ovaries showed numerous translucent and oval primary oocytes. By the end of the experiment (October '02), the male gonads appeared cream coloured and female gonads yellowish in colour with several polygonal or suboval ripe oocytes and the gametes could be easily separated when punctured. On the contrary, both meiotic I and II triploid never exhibited proper gonadal development in *C.madrasensis*. During June-July, traces of gonads were observed in both triploid groups, but the development never progressed and the squash preparation of gonad did not exhibit functional gametes. By the end of the experiment, in both triploid groups the gonads were fully shrunken and non functional (Table 6.3.4).

Condition Index: The condition index of the diploid oysters in the 3rd month varied from 16.9 to 83.3 with an average of 50.3. In the 6th month the index decreased to 39.1. By 9th month the index increased to 53.8 and in 12th the index attained maximum value of 112.5 and a minimum of 26.7 with an average of 88.7.

In the 3rd month, the condition index of II meiotic oysters ranged from 11.4 to 166.7 with an average of 63.8. In month 6 and 9, the average condition index was 66.9 and 55.3 respectively.

In the 12th month, the maximum index of 235.3 was observed with an average value of 108.5.

The condition index of I meiotic oysters showed a progressive increase from 69.23 in the 3rd month to 133.5 in the 12th month. In the 6th month, the index ranged from 11.9 to 113.9 with an average of 71.6. In month 12, maximum value of 214.7 was observed and the minimum was 92.4. The high average index value of 133.5 was observed in the 12th month (Table 6.3. 4; Fig. 6.3.2).

6.4. Discussion

Mean growth rate was higher (17.5 µm/day) for I meiotic triploids than for II meiotic triploids (15.8µm/day) and diploid larvae (12.9µm/day). The differences in larval length between diploid and both types of (II and I) triploids were significant ($P<0.05$) (Table 6.4.1). Beaumont and Kelly (1989) reported a significant increase in mean shell length of 36 days old I meiotic larvae compared with MII or control. Similarly Hawkins *et al.* (1994) observed greater growth efficiency in I meiotic triploids over II meiotic and diploids. Uttinget *al.* (1996) also recorded greater shell length in triploid larvae (103µm) than in diploid larvae (95.9µm) of the Manila clam (*Tapes philippinarum*).

Regarding larval survivability, diploids had higher (6.7%) survival than did that of II meiotic (5.2%) and I meiotic (4.5%) larvae. Low survival of triploid larvae in this study may be attributed to the quality of eggs obtained through stripping.

Growth rate in length of I meiotic triploid was 3.87mm per month, was greater than for diploids (2.99mm) and II meiotic (2.45 mm) oysters. The growth observed in this experimental study is much lower than 80-90 mm in length and 80-100g in weight obtained by Nayar (1987). This may be due to differential brood and seeds produced from stripping. The mean differences in length between the I meiotic triploids and diploids was 87.3% and between the II meiotic triploid and diploids was 53.44 (Table

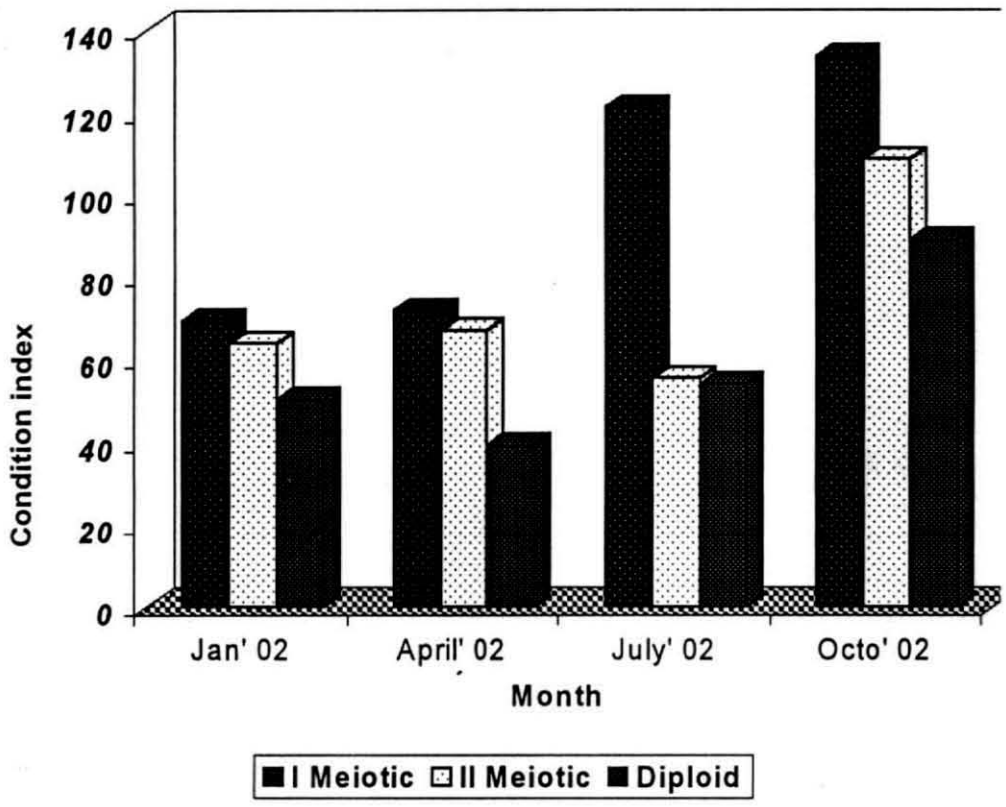


Fig. 6.3.2. Condition Index of triploids and diploid *C. madrasensis*

Table 6.4.1. **ANOVA on larval length of diploid and triploids of *C. madrasensis***

Source	Sum - of – Squares	df	Mean-Square	F-ratio	P
Treatment	592262.945	4	148065.736	429.989	0.000
Experiment	39757.797	2	19878.898	57.729	0.000
Treatment* Experiment	13076.241	8	1634.530	4.747	0.000
Error	46486.972	135	344.348		

Durbin-Watson D Statistic 1.259
First Order Autocorrelation 0.370

6.4.2). The ANOVA indicated a significant relationship between the lengths of I meiotic triploids and control ($P<0.005$) and II meiotic triploids and control ($P < 0.005$) (Table 6.4.3).

The differences in breadth ($P < 0.005$ (I M & diploid); $P < 0.005$ (II M & diploid) and width ($P < 0.005$ (I M & diploid); and $P < 0.005$ (II M & diploid) were also significant (Table 6.4.3).

Difference in shell-on weight were highly significant between I meiotic triploids and diploids ($P < 0.005$) with 260% difference (Table 6.4.2). Weight increases of II meiotic triploids and diploids was also significant ($P < 0.005$) with the difference in weight between them was 128%. Nell *et al.* (1994) also observed significant differences in morphometric characteristics between triploid and diploid Sydney rock oysters (*Saccostrea commercialis).*

AMD of I meiotic triploids was 50.5% and II meiotic triploids was 22.3% more than diploids (Table 6.4.2). The difference in the AMD between II meiotic triploids and diploids, and between I meiotic triploid and diploid oysters was significant ($P < 0.1$, Table 6.4.4). Similar observations of a high index of AMD have been reported for triploid *Crassostrea gigas* (Yamamoto *et al.,* 1988). The high AMD may be due to storage of glycogen in the adductor muscle of triploids as it, is not used for gonadal maturation (Barber and Blake, 1991). Tabarini (1984) also observed that the glycogen concentration in the adductor muscle of triploid *Argopecten irradians* could reach 135% compared with the adductor muscle of diploid scallops.

II meiotic triploids had 67% higher dry weight than that did diploids, and in the I meiotic triploids the dry meat weight was 126% more than diploids (Table 6.4.2). The ANOVA indicated highly significant ($P<0.01$) differences for dry meat weight between I and II meiotic triploids and diploids (Table 6.4.4). Nell *et al.* (1994) observed that the triploid *Saccostrea commercialis* maintained higher dry meat weights than did their diploid siblings.

Condition index plays an important role in marketing oyster meat. High index values in triploids were observed in all the

Table 6.4.2. **Differential percentage of various parameters between triploids and diploid *C.madrasensis***

Difference%		
	I meiotic & Diploid	**II Meiotic & Diploid**
Length(mm)	87.31	53.44
Breadth(mm)	60.06	39.64
Width(mm)	99.19	49.98
Total Wt(g)	260.05	128.17
Dry weight	126	67
Condition index	177.18	75.92
A.M.D (mm)	50.50	22.30

Table 6.4.3. **ANOVA on morphological traits of sacrificed oysters at different months of grow out study in triploids and diploid of *C. madrasensis***

Source	Df	Mean square							
		Length (mm)		Breadth (mm)		Width (mm)		Total weight (g)	
		I M&2n	II M&2n	I M&2n	II M&2n	I M&2n	II M&2n	I M&2n	IIM&2n
Month	3	933.848**	911.218**	888.172**	603.925**	172.358**	105.128**	418.162**	191.733**
Experiment	1	3810.002**	806.325**	970.827**	421.363**	284.504**	48.700*	588.034**	204.315**
Month& Experiment	3	285.626*	169.364	205.198*	405.854*	61.534*	20.299	160.994**	42.144**

* P<0.05, ** P<0.01

I M = I meiotic; II M = II meiotic; 2n = Diploid

Table 6.4.4. **ANOVA on Adductor Muscle diameter, Condition Index and Dry weight of triploids and diploid of *C. madrasensis***

Source	df	Mean square					
		AMD(mm)		Condition Index		Dry weight (g)	
		I M&2n	II M&2n	I M&2n	II M&2n	I M&2n	II M&2n
Month	3	112.629**	39.526**	15810.980**	89759.032 **	1.616**	58.507**
Experiment	1	264.754**	90.367**	51321.301**	135403.808 **	2.842**	59.729**
Month & Experiment	3	47.324*	7.628	3300.700	48722.036*	1.001**	25.967**

* P<0.05, ** P<0.01

I M = I meiotic; II M = II meiotic; 2n = Diploid

months over diploid siblings (Table 6.4.2). Percentage differences in condition index between I meiotic triploids and diploid was higher (177.2%) than that between II meiotic triploids and diploids (75.9%) (Table 6.4.2). The ANOVA between differences in condition index of I meiotic triploids and diploids ($P<0.005$) and II meiotic triploids and diploids ($P<0.005$) was significant (Table 6.4.4). Nell *et al.* (1994) also observed a consistently high condition index in the triploids. The higher meat condition may be due to reduced gonadal development (Beaumont and Fairbrother, 1991).

In the present study in *C.madrasensis*, triploid gonads were classified as immature or undifferentiated sex as only shrunken ovary was seen under the microscope. In triploid scallops, Ruiz-verdugo *et al.* (2000) indicated few or no eggs in a larger gonadal sac than diploids and no macroscopically visible sperm as in diploid individuals. Allen *et al.* (1986) suggested that varying DNA content and varying dosages of genetic information resulting from the random assortment of one or more chromosome as the reason for non-functional gonads in triploid fish. They also pointed out that even though triploid individuals initiate gametogenesis, it is not completed. In *C.madrasensis* also traces of gonads appeared in triploid individuals 6 months after culture, but development never proceeds as in other triploids.

Faster growth of triploids over diploids has been attributed to more heterozygous individuals having higher probability of carrying 2 or 3 different alleles in the same locus as indicated in the "allelic variation hypothesis"(Stanley *et al.*, 1984; Yamamoto *et al.*, 1988; Beaumont and Kelly, 1989). Zouros *et al.* (1996) were of the view that faster growth in triploids occurs because of the probability of exposing deleterious alleles in the homozygote state at much smaller levels. Guo and Allen (1994) suggested that nongenetic factors such as energy reallocation from gonad to somatic tissues may be responsible for faster triploidy growth. They suggested that increased cell size is the fundamental cause for triploid gigantism in molluscs. They put forward the

"cell-size hypothesis" and opined that because triploid cells have 50% more DNA, they may require more cytoplasm to maintain a given cytoplasm/nucleus ratio. Thus, triploid cells are expected to be larger than normal diploid, which has been confirmed in most organisms studied so far. Another explanation for the improved performance of the triploids is the "gene dose hypothesis" that indicates that faster growth rate in polyploids is due to triple dose of gene products. According to this hypothesis, M I and M II triploids should not differ in their growth rate. In *C. virginica* (Stanley *et al.,* 1981) and *C. gigas* (Yamamoto *et al.,* 1988; Hawkins *et al.,* 2000) M I triploids exhibited higher growth rate and increased heterozygosity compared to M II triploids. In the present study, M I triploid *C.madrasensis* exhibited higher growth like triploid *C.virginica* and *C.gigas*, but heterozygosity levels of M I & M II triploids with the loci studied were not significantly different. However, further studies are required to understand the factors that are responsible for the better growth performance of M I triploids in *C.madrasensis*.

CHAPTER 7

BIOCHEMICAL ANALYSIS

7.1.Introduction

Marine bivalves, especially the edible oyster because they are low in saturated lipids and high in glycogen and halogens, are valuable for human food. Seasonal changes in meat weight and proximate composition of an animal are associated with the reproductive cycle.

Quality and quantity of oyster meat determines the market value to the commercial growers. High quality meat for oysters is usually plumpy, creamy white and generally fills the shell cavity, whereas low quality meat is translucent, shrunken and has a relatively high water content which has been used as a more objective assessment of oyster condition (Whyte and Englar, 1982). Glycogen content is considered as a precise measure of condition of 'fatness' of the oyster (Engle, 1950).

Gametogenesis in bivalves represents a phase of particularly high demands for energy, when the transfer of proteins, lipids and carbohydrates from the adductor muscle and the digestive gland to the gonad takes place. Energy is stored as lipid, protein and glycogen before the beginning of gametogenesis, to be used later for the maturation of gametes when energy demand is high (Gabbot, 1975; Barber and Blake, 1991; Shpigel *et al.*, 1992).

Stanley *et al.* (1984) and Allen and Downing (1986) observed that gametogenesis in *Crassostrea gigas* was inhibited and that carbohydrate content decreased in diploids at the maturation peak, whereas triploids showed significantly higher levels of carbohydrates. Akashige (1990) found twice the amount of glycogen in triploids than in diploids of *C.gigas*. For the clam, *Tapes philippinarum*, higher carbohydrate content has been reported in the meat of triploids than diploids (Utting *et al.*, 1996).

Nell *et al.* (1994) compared the performance of triploid and diploid Sydney rock oysters and recorded that triploid oysters had higher glycogen content than did diploid siblings of *Saccostrea*

commercialis. Tabarini (1984) established a link between partial sterility in triploid *Argopecten irradiens* and glycogen utilization and growth. Gonadal indices of triploid *A. irradiens* were consistently lower and correspondingly, the glycogen content was consistently higher than diploids.

In this chapter, protein, fat and glycogen content in I meiotic, II meiotic triploids and diploid siblings were estimated and the results are compared and discussed.

7.2. Materials and methods

At three-monthly interval, samples for biochemical analysis were taken after measuring the morphological traits as explained in Chapter 6.

Dried samples were powdered in a mortar, transferred to a labelled polythene sachet and stored in a desiccator for further analysis.

7.2.1.Total carbohydrates

The phenol-sulphuric acid method of Dubois *et al.* (1956) was followed to estimate total carbohydrate in the sample with D-glucose as the standard. The optical density of the colour developed was measured by UV/VS spectrophotometer GBC 110 with the samples taken in a silica cuvette. The concentration of glucose in the samples was calculated (in mg%) by comparing the optical density (OD) obtained for the sample with values using the formula.

Concentrations in mg/100 mg dry tissue.

$$\left(\frac{\text{OD of the sample- O.D. of the blank}}{\text{OD of the standard-OD of the blank}} \right) \times \frac{\text{Conc. of Standard}}{\text{OD of the standard}}$$

7.2.2. Crude protein (Protein)

Nitrogen in the whole oyster was determined in 40 mg dried samples using micro-Kjeldhal technique and the values for nitrogen, were multiplied by 6.25 to estimate the protein content.

7.2.3. Total Lipids

Total lipid content was extracted following the method of Folch *et al.* (1956) using chloroform, methanol in the ratio 2:1and estimated gravimetrically.

All the values were expressed as percentage dry weight.

7.2.4.Statistical Analysis

Two way ANOVA (SYSTAT, 7.0.1) was used to compare the biochemical composition of diploid and triploid groups at four times.

7.3.Results

7.3.1.Total carbohydrates

The carbohydrate content of diploids in January was 12.65%, which increased to 14.28% in July and decreased to 12.95% in October'02. In II meiotic triploids, the carbohydrate in January was 12.65% and increased to 15.75% in July and then 18.78% in October '02. A steady increase was observed in the carbohydrate of I meiotic triploids from 13.25% in January to 20.25% in October'02 (Table 7.3.1).

7.3.2. Crude protein

Protein content of diploids varied from 78.82% in January to 78.42% in October. Whereas in II meiotic triploids, values varies from 79.13% in January to 72.05% in October. In Meiotic I triploids the protein content gradually decreased from 78.50% in January to 72.15% in October (Table 7.3.1).

7.3.3. Total Lipids

In diploids the lipid content increased from 7.62% in January to 8.15% in July and then declined to 7.75% in October. Whereas in II meiotic triploids the values increased from 7.24% to 8.55% and I meiotic triploids from 7.25% to 8.75 %(Table 7.3.1.).

Table 7.3.1. **Biochemical composition (%) of diploid and triploids of *C.madrasensis* (Mean ±SE)**

	January02	April02	July02	October02
Carbohydrate				
I Meiotic(3n)	13.25±0.23	15.65±0.21	16.50±0.07	20.25±0.63
II Meiotic(3n)	12.65±0.05	13.55±0.05	15.75±0.18	18.78±0.15
Diploid(2n)	12.65±0.07	12.76±0.11	14.28±0.18	12.95±0.03
Protein				
I Meiotic(3n)	78.50±0.09	77.1±0.12	76.00±0.10	72.15±0.10
II Meiotic(3n)	79.13±0.16	77.36±0.06	76.6±0.17	72.05±0.09
I Meiotic(3n)	78.82±0.09	78.51±0.04	77.85±0.04	78.42±0.05
Lipid				
I Meiotic(3n)	7.25±0.08	8.39±0.10	8.66±0.07	8.75±0.08
II Meiotic(3n)	7.24±0.08	8.36±0.22	8.46±0.19	8.55±0.21
I Meiotic(3n)	7.62±0.09	8.25±0.08	8.15±0.17	7.70±0.11

7.4. Discussion

In the present study, the levels of carbohydrate content showed wide differences between the three experimental groups in October. ANOVA on the differences of carbohydrate content of diploid and triploids were highly significant ($P<0.005$) (Table 7.4.1). The decrease of carbohydrate levels in October in diploids could be due to a secondary spawning during August-September. According to Ruiz-Verdugo *et al.* (2000) differences in carbohydrate between triploids and diploids are accompanied by mobilization of particular reserves to sustain reproduction. In the present study, the link between carbohydrate metabolism and gametogenesis was demonstrated by comparing the carbohydrate values of diploid and triploid in October 2002. Tabarini (1984) reported that triploid *A.irradiens* had consistently lower gonadal indices than did diploids and as a result they have higher carbohydrate content. Shpigel *et al.* (1992) attributed greater carbohydrate and protein content in triploids to lower gamete production.

The lipid content differed significantly between I meiotic triploid and diploids ($P <0.005$) (Table 7.4.1). Ruiz-Verdugo *et al.* (2001) suggested that higher values of lipid in the triploid *Argopecten ventricosus* could be due to failure of ovarian development and vitellogenesis. The higher lipid levels in I and II meiotic triploids in *C.madrasensis* may also be due to the failure of gonadal development. According to Barber and Blake (1991) during gametogenesis, several biochemical components accumulate in the gonads, providing the structural and energetic material for oocyte development. However, in triploid individuals of most mollusks, gonad development is impaired as shown by reduced gonadal indices (Komaru and Wada, 1989) or gonads exhibiting total or partial sterility (Allen *et al.,* 1986; Allen and Downing, 1986; Komaru and Wada, 1989; Guo and Allen, 1994; Cox *et al.,* 1996; Eversole *et al.,* 1996); hence mobilization of energetic components is also expected to be impaired thus showing high levels of biochemical parameters in the body. It was

Table 7.4.1. **ANOVA on biochemical composition**

Parameters	**Source of variation**	**Degree of freedom**	**Mean-square**	**F-ratio**	**Remarks**
Carbohydrate	I Meiotic& Diploid	1	210.828	763.220	P<0.005
	II Meiotic& Diploid	1	70.538	314.944	P<0.005
	I Meiotic& II Meiotic	1	37.47	176.862	P<0.005
Protein	I Meiotic& Diploid	1	121.377	1617.27	P<0.005
	II Meiotic& Diploid	1	89.274	876.471	P<0.005
	I Meiotic& II Meiotic	1	2.461	17.525	P<0.005
Lipid	I Meiotic& Diploid	1	2.090	16.257	P<0.005
	II Meiotic& Diploid	1	0.918	3.420	P<0.01
	I Meiotic& II Meiotic	1	0.238	1.090	P>0.01

also pointed out that the environmental conditions in which organisms were reared before estimation also influenced lipid content (Ruiz-Verdugo *et al.*, 2001). However, by rearing 3 groups in the same environment, greater lipid values observed in both I & II meiotic triploids than diploid in the present study may be due to a triploid effect i.e. mobilization of energetic components by partial or total sterility (Allen *et al.*, 1986; Allen and Downing, 1986; Komaru and Wada,1989).

Comparison between two groups of triploids and diploid provides evidence that biochemical composition of diploid and triploid oysters are quite different. Beaumont and Fairbrother (1991) reported that faster growth of the triploid oyster in comparison to the diploid siblings was probably due to energy saving achieved by reduced gonad development in the triploids. In normal diploids during oogenesis, primary oogonia undergo repeated mitosis to give secondary oogonia that enter meiosis I which is arrested at Prophase I. This follows the growth phase during which oocytes undergo a period of vitellogenesis, which involves accumulation of mainly lipid globules and glycogen. Triploids are generally sterile and hence ovarian recrudescence does not normally take place. The metabolic energy utilized otherwise for gonadal development will be available for increased somatic growth, thereby resulting in larger animals.

The II and I meiotic triploids of *C.madrasensis* in the present study showed significant differences in biochemical composition with I meiotic triploids exhibiting higher values for carbohydrates and lipids indicating they are superior in meat content (Table 7.4.1). Hawkins *et al.* (1994) showed that faster growth of meiotic I triploids compared to M II triploids and diploids resulted from reduced energy expenditure associated with lower concentrations of RNA per unit of total tissue protein, indicating reduced rates of protein turnover and continuous degradation and renewal or replacement of cellular protein. The physiological basis to the relationship is that increased heterozygosity enables the

triploid individual to sustain its basal metabolism with lower expenditure of energy (Hawkins *et al.*, 1994). The saving in energy may be redirected to fuel other functions such as somatic growth in *C.madrasensis* also as evidenced by the faster growth rate observed in triploid individuals.

CHAPTER 8

GENERAL DISCUSSION

Considering its suitability and commercial importance, the CMFRI evolved methods for farming the edible oyster *Crossostrea madrasensis*. Through various programmes, the techniques for oyster culture have been extended to fishermen. Good harvests led to establishment of 33 oysters farms. But harvesting period is limited to pre-spawning seasons, because meat quality decreases due to spawning. Studies were initiated in 1981 to inducing triploidy in bivalve molluscs so as to improve the quality of the meat as well as to improve production arising from their sterility. The advantages of triploidy have been demonstrated in edible oysters (Stanley *et al.*, 1981; Allen and Downing 1986; Gendreau and Grizel,1990), mussel (Yamamoto and Sugawara,1988), scallops (Beaumont 1986; Komaru *et al.*, 1988; and Baron *et al.*, 1989) and clams (Allen *et al.*, 1982; Beaumont and Contaris, 1988). So far attempts have not been made to induce triploidy in the edible oyster *C. madrasensis*. In the present study, attempts were made to develop suitable induction methods for *C. madrasensis*, and the growth and meat weight of triploids and diploid siblings, and the performance of I and II meiotic triploids were compared.

One of the prime requisites for triploidy induction is the time at which 50% of fertilized eggs develop polar bodies (Allen *et al.*, 1982). In this study 50% of I polar body formation was observed at 16 minutes at 29°C. At 23°C it took 18 min and 15min at 31°C respectively. Development of II polar body occurred at 36, 32 and 28 min at temperature of 23, 29 and 31°C respectively. Quillet and Panelay (1986) also observed critical periods for effectiveness of thermal induction were 10 and 15 min and 35 min after fertilization. Desrosiers *et al.* (1993) treated 6-DMAP, 15 min after fertilization for 90% of triploid production in *C. gigas*. For *C. madrasensis* the critical period for production of triploids is 16 minutes and 32 min post fertilization at an ambient temperature of 29°C. Gerard *et al.* (1994) also observed 50% polar

body extrusion in 15 min post fertilization in *C. gigas*. The kinetics of polar body extrusion is inversely related to temperature. Hence for production of I meiotic triploid, inducement may be given 8 min after fertilization, and for II meiotic triploids 17 minutes after fertilization.

Physical and chemical methods have been employed for induction and production of triploids. In this study heat and cold shock physical methods, and CB and 6 -DMAP chemical treatments were trialed. Of these, 6-DMAP (100 µM) yielded 66.6%, cold 42.25% and heat and CB (0.05 mg/l) yielded 40% triploidy. The 6-DMAP treated eggs of *C. gigas* gave 90 – 95 % triploidy (Desrosiers *et al.*, 1993). As the 6-DMAP treatment does not require equipment, is easily dissolved in seawater and not as toxic as CB, 6-DMAP can be effective for production of triploidy in *C. madrasensis*. Moreover, 6-DMAP is a universal inducer in *Mytilus edulis, Placopecten magellanicus*, and *C. gigas* (Desrosiers *et al.*, 1993), this chemical can therefore also be effective in *C. madrasensis*. Comparatively low triploid percentage (66.6%) was obtained by 6-DMAP treatment in this study. The inability to get cent percent triploid by 6-DMAP treatment may be due to the inappropriate treatment time and duration. Liu *et al.*,(2004) suggested that the timing of 50% PB I extrusion may not be effective to obtain higher percentage of triploids. Hence attempts have to be made in future to upgrade the triploid inducement techniques in *C.madrasensis*, considering the triploid induction factors i.e. dosage, starting time and treatment duration as indicated by Liu *et al.*, (2004) in production of triploids in molluscs.

Further studies have to be undertaken to assess the effectiveness of cold shock for commercial application as an alternate to chemical method, which is considered unsafe.

Effective dosage of 0.05mg/l CB produced 40 – 41.8% triploids in this study. Among the six agents CB was recognized as the best inducer producing 86% of triploids in *M.*

galloprovincials (Scarpa *et al.*, 1994) and 67.2% in *C. gigas*. Being carcinogenic, the usage of CB is restricted. To avoid noxious physical or chemical treatments, mating tetraploids with diploids to produce triploids is the best alternative, as suggested by Chew (1994).

The faster growth of triploids facilitates higher production. It reduces 20% of labour cost by reducing the grow out time (Graham, 1991). In the present study, significant morphometric differences were observed, and 87.3% differences in the length between I meiotic and diploid ($t = 6.92$; 10; $P<0.01$) and the difference between II meiotic and diploid ($t = 4.02$; 8; $P<0.01$) were highly significant (Table 8.1). The difference in weight, i.e. 260% and 128% for I and II meiotic to diploid respectively were also significantly different (5.13; 8; $P<0.01$).

Highly significant difference in dry meat weight between I meiotic and diploid (18.29; 7; $P<0.01$) with regard to the condition index were observed (Table 8.1).

In the present study direct comparisons of the percentage of differences in commercially important aspects such as total length, total shell on weight, dry meat and condition index among diploids, I & II meiotic triploids; the I meiotic triploids always had higher percentage, of all these factors. This indicated that the performances of I meiotic triploids are better than II meiotic triploids.

Stunted growth of gonads was seen in triploids while in diploids both ovaries and testis were functional. Further histological observation will be needed to study the annual variation of gonads in triploid individuals of *C.madrasensis*.

Higher levels of total carbohydrate (including glycogen) 20.25% in I meiotic triploids and 18.78% in II meiotic triploids over 12.95% in diploids has a major role in increasing the superior performance of triploids. Higher values of lipid, 8.75% in I meiotic triploid and 8.55% in II meiotic triploid over diploid (7.70%) adds texture and flavour of triploids.

Table 8.1. **'t' test for various parameters between triploids and diploid *C.madrasensis***

Parameters	Groups	t - value	df	Significant level
Length(mm)	I Meiotic & Diploid	6.92	10	P<0.01
	II Meiotic & Diploid	4.02	8	P<0.01
Breadth(mm)	I Meiotic & Diploid	5.42	8	P<0.01
	II Meiotic & Diploid	3.75	7	P<0.01
Width(mm)	I Meiotic & Diploid	5.16	11	P<0.01
	II Meiotic & Diploid	2.79	6	P<0.05
Total weight(g)	I Meiotic & Diploid	5.13	8	P<0.01
	II Meiotic & Diploid	2.04	4	P<0.05
AMD(mm)	I Meiotic & Diploid	18.29	7	P<0.01
	II Meiotic & Diploid	1.66	3	P<0.1
Dry weight(g)	I Meiotic & Diploid	13.06	7	P<0.01
	II Meiotic & Diploid	4.32	4	P<0.01
CI	I Meiotic & Diploid	2.79	6	P<0.5
	II Meiotic & Diploid	1.33	3	NS

All the allozymes used in the present study were useful as markers for diagnosis of ploidy in the species because they allowed direct visualization of gene duplication at discrete structural gene loci. After allozyme electrophoresis it was also observed that heterozygosity was higher in triploids for all 10 loci. Though differences were significant between triploids and diploids ($P<0.0001$), there was no difference in heterozygosity between meiotic I & II triploids ($P>0.05$).

Better growth performance of I meiotic triploids over II meiotic triploids and diploid was observed in *C. virgnica,* (Stanley *et al.*, 1984) *C. gigas* and *M. edulis* (Yamamoto *et al.*, 1988). Hawkins *et al.* (2000) suggested that the increased heterozygosity of meiotic I triploids may be due to sustaining basal metabolism with less expenditure of energy.

In *C. madrasensis* also, it has been observed that I meiotic triploids are advantageous in growth performance than II meiotic triploids suggesting that higher concentration of I meiotic triploids may be attempted in further studies.

Meiotic I triploids which were significantly bigger than meiotic II triploid groups in *C.madrasensis* did not show increased heterozygosity at all the 10 loci analysed. There was no detectable or real influence of heterozygosity *per se* on the body size within both types of triploids in the present study. It may be explained by the "associative overdominance hypothesis" that probably other genes (rather than those examined here) are responsible for differences in body size (Beaumont *et al.,* 1995). However further studies are required to support the above mentioned hypothesis in *C.madrasensis.*

The additional cost in production of 1 million triploid oyster seed will be Rs.1000/-(US $ 24) more than that of diploid oyster seed. The cost increase is the amount of 6-DMAP required for triploidy induction. With the available facilities in a molluscan hatchery, no additional effort is involved in the 6-DMAP treatment

process. Hence the cost of production of triploid seed will be very minimal.

Muthiah *et al.,* (2000) estimated production of diploid oyster as 80 t/ha using rack and string method. The 126% dry meat weight increase in triploids will tend to increase the production of oysters. Thus the yield could be increased to 100 t/ha by culturing triploids by the oyster farmers.

As the growth rate of triploid *C.madrasensis* (12.9mm/day) is more than that of diploids, the reduction of farming duration from 12 to 7 months will facilitate better farm management, hereby reducing the production cost of oysters.

However, since the triploid *C.madrasensis* having two fold increase in glycogen content than diploids, meat quality will be better and accepted by customers. This will help the farmers in marketing their product.

Hence to obtain 100% triploids apart from physical and chemical treatments, mating tetraploids with diploids to produce triploids can be the best alternative as suggested by Chew (1994) and Guo and Allen (1994). Studies on reproductive characters of tetraploid *C.madrasensis* have to be undertaken so that crosses of tetraploids with diploids will yield 100% triploids which could be supplied to farmers to increase oyster production.

CHAPTER 9

SUMMARY

The salient points of the work are summarized below

The kinetics of extrusion of polarbody is essential to produce triploids. Hence, studies were conducted on polarbody extrusion at 22, 28 and 31(±1)°C. At 31,28 and 23°C, 50% of I polar body was formed 15, 16 and 18 minutes respectively after fertilization. At 31, 28 and 23°C, 50% of II polar body was formed at 28, 32 and 36 minutes respectively after fertilization. Hence triploid induction treatments were administered 8 minutes after fertilization for producing I meiotic triploids and 17 minutes after fertilization for II meiotic triploids.

Among physical methods of induction for triploidy, cold and heat shock treatments were applied. Cold shock at 5°C for 10 minutes duration yielded 42.25% of triploidy.

High percentages 42.0 and 41.85 of triploids occurred at 37°C for heat treatment, for 5 and 10 minutes, respectively.

In the chemical treatment, among three concentrations of cytochalasin B (0.05, 0.10 and 0.15 mg/l), 40.0% triploids were obtained in 0.05mg/l of CB treated fertilized eggs.

Another chemical 6-Dimethylaminopurine (6-DMAP) at 50, 100, 150 and 200μM was tried at various treatment durations of 5,8 and 10 minutes. High percentage of 66.6 triploids was obtained from the fertilized eggs treated in 100μM concentration of 6-DMAP for 8 minutes.

Among the treatments applied for production of meiotic triploids, 6-DMAP (100μM) yielded high percentage (66.6) of triploids followed by 42.3% by cold (5°C) treatment and 40% in heat (37°C) and CB (0.05mg/l) treated eggs.

Polyacrylamide gel electrophoresis was carried out to assess allozyme variation of Esterase, Phosphoglucomutase, Glucose-6-phosphate dehydrogenase, Glucosephosphateisomerase

and Superoxide dismutase. All these allozymes were found to be ideal markers to detect triploidy in *C.madrasensis*. Heterozygosity was higher in triploids for all the ten loci examined.

The growth rate in length of I meiotic triploid oysters was 3.87 mm/month whereas diploid and II meiotic oysters registered 2.99 and 2.45 mm. The differences in length between I meiotic triploid and diploid was 87.3%. In shell-on weight also high percentage of 260 was observed between the I meiotic triploids and the diploid. The adductor muscle diameter was 15.5mm in I meiotic triploid whereas it was 9.8mm in II meiotic triploids and 5.6 mm in diploid siblings.

Higher dry meat weight of 1.45g was observed in I meiotic triploids compared to 0.86 and 0.19g in II meiotic triploids and diploids respectively. With regard to condition index, high value of 133.5 was registered for I meiotic triploid oysters than 108.5 for II meiotic triploids and 88.7 for the diploids.

Gonadal development did not progress in both meiotic I & II triploid individuals in *C.madrasensis*. At 12 months of culture, the diploid individuals exhibited ripe ovaries and testis while triploid gonads were classified as immature or nonfunctional.

The carbohydrate content of 12 months old I meiotic triploid *C. madrasensis* was 20.25% whereas II meiotic triploids had 18.78%. In the diploid siblings, because of occurrence of spawning, the carbohydrate content was 12.95% only. The lipid value was 8.75%, 8.55% and 7.70% in I meiotic, II meiotic and diploid respectively.

Treatment of fertilized eggs of *C. madrasensis* in 100µM of 6-DMAP for 8 minutes for obtaining high percentage of triploids. High growth rate, more dry meat weight, high carbohydrate and lipid content, condition index and non-functional gonads of I meiotic triploids indicated the advantages in farming the triploids to augment higher edible oyster production.

Further experiments are required to study the effect of induction factors, (dosage, starting time and duration) for inducement of triploidy using 6-DMAP to increase higher hatching rates in *C.madrasensis*.

Further studies are also important to understand the relation between heterozygosity and growth in I meiotic triploids.

Studies in future have to be oriented for developing techniques for production of tetraploid in *C.madrasensis* in order to produce 100% triploid oysters by crossing tetraploid with diploid individuals.

CHAPTER 10

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Appendix A

Staining recipes used for isozyme analysis

Esterase EST. E.C.3.1.1.1 (Monomer)

α and β Napthyl acetate (20mg each in 0.5 ml acetone +0.5 ml H_2O)	-	0.5 ml each
FRR 5mg/ml	-	5ml
Tris HCl Buffer (pH 7)	-	2.5ml
Double Distilled Water	-	6.0 ml

First pour the substrate over the gel and then stain.

Glucose – 6 -Phosphate Dehydrogenase G_6PDH. E.C.1.1.1.49 (Dimer)

0.2M Tris HCl (pH 7.1)	-	2 ml
Double distilled water	-	15ml
Na_2 Glucose $_6$ PO_4	-	0.09g
NADP	-	1.1ml
* NBT	-	750µl
* PMS	-	0.2ml

Incubate at 37°C in the dark.

Superoxide dismutase SOD. E.C. 1.15.1.1 (Dimer)

0.2 M Tris HCl, (pH 8.5)	-	15 ml
*MTT 0.5%	-	0.5ml
*PMS 0.5%	-	0.5 ml

Incubate at 37°C in dark until activity just begins to appear and then set in indirect light until sufficient activity was present.

Phosphoglucomutase PGM. E.C. 5.4.2.2 (Monomer)

Glucose 1Phosphate (50mg/ml)	-	1.0ml
NADP (4mg/ml)	-	1.6ml
$MgCl_2$ (20mg/ml)	-	1.0ml
**G_6PDH (0.5 ml make salt)	-	20µl
NBT (8mg/ml)	-	0.4 ml
*PMS (1.7 mg/ml)	-	0.2 ml
Tris-HCl (pH 8.0)	-	2.5 ml
Agar Over lay	-	2 %

**Sigma 1000 units – Pour 1ml Double Distilled Water.

Glucose Phosphate Isomaerase GPI. 5.3.1.9 (Dimer)

Fructose 6 Phosphate (20mg/ml)	-	1ml
NADP 4mg/ml	-	1.6ml
$MgCl_2$ 20 mg/ml	-	0.5 ml
G_6PDH 0.5 ml	-	20µl
*NBT 8mg/ml	-	0.4ml
*PMS 1.7mg/ml	-	0.2 ml
Tris- HCl (pH 8.0)	-	2.5 ml
Double Distilled water	-	3.8 ml
Agar Over lay	-	2%

Incubate at 37°C in the dark.

***Add when ready to stain**